Brain protein metabolism following imprinting in the chick

Thesis

BRAIN PROTEIN METABOLISM FOLLOWING IMPRINTING IN THE CHICK

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Date of submission 1·11·81

Date of award 1·4·82

This thesis is submitted to the

Open University in part fulfilment

for the degree of Doctor of Philosophy

Alan Longstaff
1981
Aberdeen

To my mother

For her love, encouragement and support

' They brynge vp a greate multytudè of pulleyne, and that by a meruelous policie. For the hennes doo not syt vpon the egges: but by kepynge them in a certayne equall heate, they brynge lyfe into them, and hatche them. The chyckens, assone as they come owte of the shell, followe men and women in steade of the hennes.'

Sir Thomas More

(Utopia 1516, translated from the Latin by Robynson in 1551)

ACKNOWLEDGEMENTS

Although the synthesis is my own, this work could only have been achieved as a result of the contribution made by others.

Foremost is Professor Steven Rose, who I should like to thank for his invaluable guidance and encouragement.

Jeff Haywood, by his example taught me a great deal about research techniques. For their advice about various aspects of this work, I should like to acknowledge Pat Bateson and Joe Schwartz.

For their technical skills I am indebted to Hilary Bolton for her part in designing and running the automated AChE assay, to Tony Cassidy for his help in the design and building of the imprinting equipment and to Tommy Hedges, Steve Walters and the staff of the animal house for their continual care of the chicks.

I am especially grateful to Elaine Dalgarno for her painstaking secretarial work.

These and members of the Brain Research Group provided not only discussion and critique which has inevitably influenced my thinking, but also a constant basis of friendship and support through what has at times been a 'maturing experience'.

CONTENTS

ABSTRACT

Imprinting, a type of learning in which neonatal precocial birds come to form long lasting social attachments to visually conspicuous moving objects has a number of biochemical concomitants, including region specific increases in the synthesis of RNA and protein. The results of a series of experiments when taken together, indicate that the altered nucleic acid synthesis could be a correlate of learning. In part, the present work seeks to establish how closely the modulations in protein metabolism are related to learning and to gain some insight into the nature of the proteins involved. In addition, the possible implication of cholinergic mechanisms in learning has been explored.

Over the twenty minute period following exposure of young chicks to a flashing light as an imprinting stimulus, there is an increased incorporation of ^{14}C leucine into an acidic protein fraction of the anterior dorsal forebrain. This brain region has been implicated in several studies as the locus for a number of biochemical modulations that accompany learning. The amount of ^{14}C leucine incorporated does not seem to be determined by precursor pool availability, nor is it simply the result of visual stimulation. It does, however, correlate with a well validated measure of the extent to which the birds have learnt to recognise the characteristics of the stimulus, as shown by a two choice discrimination test. There is no change in the total content of tubulin dimer as assayed by colchicine binding under these conditions.

While no modification of acetycholinesterase activity was detectable following imprinting, dramatic changes in muscarinic receptor levels, as assayed by atropine displaceable ^{3}H-quinuclidinyl benzilate binding, were found in posterior dorsal forebrain and midbrain of birds which showed evidence of having been imprinted compared to those which had apparently failed to learn the stimulus characteristics.

PUBLICATIONS

The acetylcholinesterase study has been reported in Longstaff, Schwartz and Rose (1976). Protein synthesis data from the 2 day experiment has already been published (Longstaff and Rose 1978) and a full communication of all the findings relating to the 2 day experiments is currently in press with J Neuro chem (Longstaff and Rose 1981).

INTRODUCTION

Imprinting is a behaviour, described originally in precocial birds, in which the neonate learns to recognise the characteristics of a visually conspicuous moving object and to which it becomes socially attached. This work sets out not only to describe data which represents biochemical correlates of this particular behaviour, or simply to justify the findings with respect to other studies which have used the same or similar paradigms, but to view the neurochemistry of imprinting from a far broader perspective. In so doing the underlying theme has been to argue from the general to the particular and while, hopefully, supportive experimental evidence has never been too remote in such discussions, where it has seemed warranted I have not declined to speculate, though the intention has always been to differentiate clearly between fact and conjecture.

An overview of the chemistry of learning (Chapter 1) provides a framework, which although by no means exhaustive or comprehensive, is designed to highlight specific questions, to pinpoint ways in which these have been tackled, and to signpost the way in which model building might be most expeditious. In the past insufficient emphasis has been given to the importance of the relationship between learning and affective states. What is needed now are more studies which are concerned with these epiphenomena at the molecular and cellular level, so that it becomes possible to recognise them in biochemical and physiological terms when they 'contaminate' learning studies. Where it has seemed appropriate the discussion has been enlarged to include these eventualities. Where necessary to the argument treatment of some evidence has been relegated to consideration later in the thesis and this is especially the case with imprinting neurochemistry which provided the background to the present studies; for the sake of clarity this material is treated en masse (Chapter 3). The similarity of the neurochemical response seen in chick imprinting to that shown in learning situations generally, in a variety of species, is fairly striking and suggests either that global models are quite justified, or that our approaches so far have been too crude to make the necessary discriminations.

Necessarily the use of any particular behaviour in the study of a more general phenomenon warrants vindication and this is especially true for imprinting, the nature of which has been misconstrued. In the past some workers have regarded it as a unique behaviour, distinct from learning, and others as a special type of learning peculiar to precocial birds. Clearly it may not be expedient to study what is unique or peculiar if the search is for a model of more generally applicability and the assumption about which most of the research discussed later pivots

is that underlying all memory mechanisms is a common phenomenology. That is to say, the plurality apparent in learning reflects semantic, artifactual or contextual features rather than any fundamental distinction. The second chapter sets out firstly to demonstrate that because certain notions about imprinting are no longer tenable it can be profitable to study its biochemistry and secondly to analyse aspects of imprinting behaviour which may be significant in the design of suitable methodologies.

The core of the thesis is devoted to detailing the experimental techniques used and in discussing the data which this generated and how it may be interpreted.

While the evidence for the involvement of RNA synthesis in learning the imprinting stimulus characteristics is rather good, the role of the protein synthesis, and AChE activity changes with imprinting described previously is more problematic. The purpose of the present studies initially was to determine how closely related these modulation were to learning per se, rather than the contingent light exposure, and to gain some insight into the nature of the proteins involved.

CHAPTER 1

THE NEUROCHEMISTRY OF LEARNING AND MEMORY

1.1 Plasticity

In the developing visual system of the chick, optic nerve fibres give rise to an ordered projection across the tectum before the formation of retino-tectal synapses (Crossland, Cowan and Rogers 1975). This is an example of a universal process termed neuronal specificity; the exact mechanism of which is far from clear, but which is assumed to be genetically predetermined. A nervous system exclusively 'hard-wired' in such a fashion would result in an organism capable of only purely stereotyped behaviours and which would be totally non-malleable in the face of environmental exigencies. The evolution of an animal with the capacity to modify its neural output pattern or behaviour as a result of experience must have occurred very early on, in phylogenetic terms, since the ability appears ubiquitous and moreover this plasticity would have conferred an overwhelming selective advantage on its possessor. While such experientially dependent modulation is not genetically programmed per se it is to be assumed that the units within the nervous system responsible for that plasticity are genetically destined to be plastic.

In the cat the pre-requisite for the normal development of the visual cortex is normal visual experience during the first few months of life. In other words during the ontogeny of the cat CNS sensory input modifies the characteristics of particular arrays of neurones in such a way as to alter their output. A key point about this process is that the visual experience must occur during a critical period of development which suggests that the neuronal arrays in question are initially labile but as maturation proceeds they become fixed, and furthermore any programming by the environment is essentially irreversible. It is argued on the basis of biological parsimony that this ontogenetic plasticity is echoed in the phenomenon of learning. By learning is meant the predictable modification of behaviour which follows a particular and rapid experiential change. In general terms it seems that learning is a more flexible process than environmentally wrought alterations during development. Learning is usually not restricted to a critical period in the life of an animal but may occur at any time. What this implies is that those neuronal arrays concerned with the plasticity of learning remain in a labile state until they are modified by the learning experience. Further, learned habits suffer extinction, but this does not necessarily mean that a particular neuronal array remains forever labile, for the

learning modulations to be switched on and off as environmental caprice dictates; more likely learning plasticity involves relatively permanent irreversible modification, as does ontogenetic plasticity, and extinction comes about by similar means in a separate neuronal array. However, learned habits are also prone to forgetting, which may be because either the plastic units involved are not stable and may revert with time to their original state (although self evidently this does not occur in all cases) or that the storage form becomes unavailable for recall. Finally recall clearly plays no part in ontogenetic plasticity. Despite these differences it may be assumed for reasons of evolutionary economy that the molecular and cellular events that underlie all plasticity are common, and differences arise because of the number, addresses and connectivity of the cells (i.e. the particular array) involved.

Exactly how the changes in synaptic connectivity which represent plasticity are achieved at the morphological, physiological and biochemical levels should be the objective of any global model which attempts to provide an explanation for learning in cellular terms, and must account for acquisition, storage, recall and forgetting. Historically, the pursuit of biochemically specific processes has needed to explain both the overwhelming evidence that memory has at least two components, and that at some time shortly after the initial acquisition the transient short term memory (STM) must be consolidated into the long term store (LTM), if recall much later is to be possible (but see Lewis 1979 for a rather different interpretation). For a short period after acquisition subsequent recall can be readily disrupted by a wide variety of interventions such as hypoxia, hypercapnia, anaesthesia and electroconvulsive shock (ECS); though the exact time over which ECS, for example, is amnestic varies with the task, the species and the experimenter (Chorover and Schiller 1965), and can be protracted by cooling (Agranoff, Davis and Brinks 1965). Later these insults have no effect on recall, and once consolidated LTM is resistant to almost anything except massive lesions. On such a basis it can be argued that short and long term storage modes are biochemically distinct. Consolidation seems to be dependent on transcription and translational processes, which self-evidently must be tightly linked either serially or in parallel with the events representing STM. Consolidation is also selective, as not all behaviours acquired in the short term are retained.

1.2 Experimental design

However learning may be defined on a theoretical basis, operationally - that is within the context of an experiment - it can only be determined by some measure

of performance. That is, an experience which is imposed on an animal should generate a subsequent precise modification in the behaviour of that animal; a predictable response produced to a well defined set of environmental circumstances. Since performance is influenced by motivation, the degree of stress, the level of arousal and many other factors, a key problem is to differentiate between those biochemical changes that correspond to learning per se from those which are influences of associated processes. Of course such a separation may be artificial. Clearly, in order to learn an animal has to be attentive to stimuli and aroused, and to dissect the effects of learning from those of attention and arousal may be quite arbitrary, though attempts to do just this have been made. For a review of these problems see Rose 1981.

At least in the life sciences and perhaps more especially in neurobiology the majority of experiments are ambiguous. Ambiguity may arise because confounding variables either were not or cannot be adequately controlled for within a single experiment. In learning experiments even the 'yoked' control may not be perspicuous, for such an animal may learn, for instance, that it cannot escape from an aversive stimulus, and might also consequently suffer greater stress than the experimental animal. Often a paradigm may be manipulated in several different ways so that while any one study may have several interpretations, collectively, a 'balance of probabilities' is established which favours one in particular (see, for example, the work which has implicated RNA synthesis following imprinting in the chick as being necessary for learning). Clearly this is a less than completely satisfactory situation but may be the best possible at present. An additional problem is whether learning is a unitary phenomenon; that is, can the biochemical findings in a learning experiment conducted in one species be translated to other species. Although this is the assumption implicit in most reviews it is clear that just as there are interspecies differences in sensory processes, so are there likely to be differences in the manner in which such input is treated within the CNS.

1.3 Learning models

At another level difficulties may arise because the theoretical constructs are themselves flawed. Evidence can be adduced in favour of both perceptual learning or conditioning theories of imprinting, but may be there is no a priori reason to regard these as mutually exclusive viewpoints; perhaps the situation is reminiscent of the wave/particle duality in physics, and the paradox arises partly because in the past experimenters have chosen to explore too restrictive a set-up. The demise of Skinnerian behaviourism which purported to be an exclusive description

of learning was surely greatly hastened as psychologists 'discovered' that pigeons and rats could behave in rather more versatile fashion outwith the confines of the Skinner box. So desperate has been the need to describe learning in associative terms it has even been postulated (Mackintosh 1973) that habituation arises as a result of a stimulus reinforced by nothing of consequence happening to the animal. From this it follows that no stimulus can be unreinforced and the term latent learning as defined by Thorpe takes on a somewhat different meaning.

Such eccentricities aside, notions about the role of reinforcement in associative learning have changed considerably in the past few years. Conditioning may be regarded as brought about not by contingency but by correlation. One particular experiment (Rescorla 1967) illustrates this well. Two groups of rats given identical CS - US pairings (tone-shock) obviously experience the same contiguity of pairings, but if one group also experiences randomly distributed tones unpaired with shock, i.e. the correlation between tone and shock is worsened, then learning is found to be relatively impaired. Neither is reinforcement brought about by a particular class of elements but is a relationship between two behavioural events, so although rats will run for a drink reward under one set of circumstances, they can also be induced to drink in order to run under different conditions (Premack 1965). Furthermore reinforcement need not be immediate. Illness (CS) can be induced in chicks even 24 hours after a single experience of drinking a sucrose solution and still be an effective negative reinforcer (Gaston 1977). In this case the appropriate clue (US) is visual. It is argued that for recall to occur the behaviour which is subsequently to be reinforced has to be flagged in some way. As an illustration of this Lett (1973) has shown that removing a rat from a maze flags the last response that the animal made, and returning it to the maze after an interval to be rewarded enables it to relate the flagged behaviour with the reinforcement. Clearly learning in this case occurs in the interval between the CS and the reinforcement and seems to be effected by a contextual change (i.e. removal from the maze). This is reminiscent of perceptual learning, which implies a mismatch between previously established percepts and current experience, and has two consequences. Firstly it becomes more difficult to argue that control animals do not learn since reinforcement seems important only for recall (which may be determined by motivation) and not for acquisition. Secondly the possibility emerges that the prime factor in the acquisition of learning is change. In the way that the responsiveness to motion of units in the cat visual system may be held to underlie the fact that moving features in the cats percept have greater significance than static ones, so whatever plastic modifications underlie learning are brought about by a changing rather than a constant environment, because the former has greater significance. By significant is meant

those features resulting in behavioural arousal and attentiveness. Moreover, in the same way that an alteration in the external context may flag a behaviour, so that return to the original context may enable recall, an alteration in the internal context (i.e. physiological state) can do likewise. What is interesting is that many of the drugs capable of effecting such state dependent behaviours are hypnotics which alter arousal levels, in this connection it is noteworthy that Wells (1974) regarded sensitization, with its period of enhanced responsiveness to stimulation following reward or punishment as the precursor of association learning. Here the significant event would seem to be the reward/punishment and the assumption implied is that enhanced responsiveness and arousal are analogous.

At the physiological level the interaction between arousal and learning presumably first requires that a particular assemblage of cells responds to a specific pattern of sensory inputs by initiating the processes involved in short term memory (STM). This same network of cells will receive an input from some general arousal system, the level of which will be controlled by the global sensory experience of the animal at that time and the significance that the specific pattern of inputs has for the animal; thus, should the input be rewarding or aversive then the level of arousal will be correspondingly high.

Since the early work of Olds and Milner (1954) it has become clear that an animal will work to receive electrical stimulation of a number of discrete sites in the brain. Such sites have come to be regarded as part of a reward system. Thus, stimulation of the lateral hypothalamus in the rat appears to mimic a food reward being provided to the animal (Rolls 1974). The reward system neurones receive input from two sources, one informing about the physiological state which translates as hunger, the other provides information about the presence of food, and on this basis the system will determine the reward value of food and so enable the appropriate behavioural response.

Interestingly it appears that arousal may in fact be produced as a result of rewarding lateral hypothalamic stimulation, through activation of neurones in the brain stem and medial posterior thalamus. Evidence for this is that with lateral hypothalamic stimulation EEG desynchronisation occurs which decays over several minutes (Rolls 1974), and in the unanaesthetised rat this shows itself as stimulus related locomotor activity; an effect that can be mimicked by the amphetamines (Kelly, Rolls and Shaw 1974).

So, it appears that reward can generate arousal. However, it will be recalled that change per se may be significant and so generate learning, and presumably here too, despite the absence of overt reinforcement, there may still be mediation by the reward system which presumably continually 'monitors' the hedonic state of the environment (measuring significance) and adjusting the affect of the animal accordingly effects appropriate learning acquisition.

Given the practical and theoretical difficulties outlined above any neurochemistry of learning must be able to incorporate knowledge of affective states into its domain, and the remainder of this chapter is devoted to a more detailed examination of the evidence which has helped to shape learning models in biochemical terms. As yet the ideas are incomplete and often vague, reflecting perhaps the comparative crudity of the methodologies employed and the lack of any sure foundation in psychology.

1.4 Sensory stimulation and cerebral blood flow

As early as 1 hour after monocular eye suture in day old chicks there is reduced blood circulation through the contralateral as compared with the ipsilateral brain regions (Bondy 1973). This occurred only transiently in the cerebral hemispheres, but was still present after 15 days in the optic tectum. The effect was reversible in the sense that if the sutures were removed, even after as little as 1 hour there was an increase in blood flow to the opposite side. Such a phenomenon is not unique to birds; the visual cortex of cats responds in a similar way to retinal illumination (Sokoloff 1961).

Chicks monocularly sutured immediately after hatching and which had received no prior light exposure showed a greater increase in contralateral blood flow when the sutures were removed at two days than birds which had experienced light previous to suturing. Bondy and Purdy (1976) argue that the increased blood flow may be a pre-requisite for imprinting, but they also obtain differences in chicks after 7 days which they believe militates against such a view because chicks are no longer able to imprint at this age (Hess 1964). However, as will be argued later, Hess takes a too rigid view of the sensitive period.

The finding that a previous short period of light exposure improves both passive avoidance learning (Cherfas 1977) and imprinting (Bateson and Wainwright 1972; Bateson and Seaburne-May 1973), does highlight a possible role for increased cerebral blood flow in optimising these behaviours. The picture is not a simple one and factors other than blood flow are implicated, because it has been found that it is patterned light that is necessary for 'activation' of the visual system. Maybe this is because the majority of retinal units are phasic and would rapidly habituate to a homogenous visual field (Cherfas 1977). Supporting this general view the impairment to the learning of visual tasks brought about by intracranial cycloheximide (CXM) does not occur in birds kept in the dark or exposed to non-patterned light (Rogers and Drenen 1978).

Light exposure per se is not the only determinant of cerebral blood flow. In an experiment where chicks pecked at grain they could only see with one eye, while the contralateral eye received identical light stimulation, the optic lobe and hemisphere which attended to the grain had significantly greater blood flow. Since the mechanics of grain pecking is not learned it was reasoned that the changes were a result of attention or arousal (Bondy, Lehman and Purdy 1974). However while it may be the case that pecking itself is innate it seems likely that while executing the behaviour the animal is learning, e.g. to improve its motor co-ordination and so to peck more accurately, and to discriminate between food and inedible material.

If one expression of learning/attention/arousal be enhanced neuronal activity it would be expected to create greater metabolic demands requiring increased uptake of precursors from the extra-cellular fluid and blood, and a concomitant increase in the levels of vasoactive metabolites would presumably be responsible for altering regional blood flow. While such, presumably anatomically specific, alterations in capillary bed perfusion are remarkable, it seems unlikely that these changes can be other than permissive, and an analysis of phenomena more likely to be directly related to learning is warranted.

1.5 Early events in learning

Within a few hours of hatching chicks will peck at any small object which contrasts

sufficiently with the background, and in a single trial can learn to refrain from pecking a coloured bead coated with the unpleasantly tasting substance methylanthranilate (MeA). The task is discriminatory, in that a bird so trained will continue to peck at non-tainted beads of a different colour. This passive avoidance learning, utilized by Gibbs and her colleagues (reviewed in Gibbs and Ng 1977) seems particularly appropriate for an appraisal of early biochemical events in learning using various inhibitory drugs. Such work led to the proposal of a three stage model for learning.

One trial learning can be impaired within 5 minutes of acquisition by the bilateral injection of non-physiologically low or high concentrations of K^+ or Na^+ into the chick forebrain, either 5 minutes before or up to 2.5 minutes after the training trial. It is interesting that 140 mM Na^+ or 7 mM K^+ are without effect, since these are just the ionic concentrations of chick CSF (Anderson and Hazelwood 1969). It was postulated (Gibbs and Ng 1977) that neuronal activity gives rise to a potassium ion mediated hyperpolarization which inhibits subsequent impulse propagation and so serves to preserve the recent firing history of the neurone from ionic disruption until it is translated into a more stable form. The finding that Ca^{2+} ions prolong this STM phase might be important since it is known that it increases the duration of hyperpolarization produced by elevated K^+ conductance. Calcium ions then may underlie the maintenance of STM.

What is being proposed essentially is that those neurones involved in acquisition soon cease firing. If recall is brought about through the same neurones (anything else would seem unparsimonious) then a transient learning deficit is predictable within the first few minutes, presumably ended only by the cells returning to their normal polarized state after the memory has been fixed into its more stable form.

However, Kamin deficits in the performance of learning tasks which may result from retrieval defects occur within 1-6 hours of acquisition but not, for example, at 3 minutes (Bryan and Spear 1976).

A short while later is the so called labile phase in which 1 or 4 mM K^+ are still effective inhibitors, but now the inhibition can be prevented by the administration of diphenylhyantoin (DPH), amphetamine and noradrenalin (NA) suggesting that the low potassium concentration is inhibiting the cation pump. This view is strengthened by finding that learning deficits are produced by sodium pump

inhibitors such as ouabain and copper (Watts and Mark 1971) which interfere with Na^+-K^+ ATPase (Rang and Ritchie 1968). The action of ouabain is not attributable to acquisition deficits, since administering the drug after the training trial had the same effect as giving it beforehand; memory impairment not manifesting itself until 15 minutes after training. Since retention is normal for the first 15 minutes the drug would not seem to be affecting retrieval mechanisms or STM. When sodium pump inhibitors were given later than 10 minutes after the training trial however no deficit was observable, suggesting that this phase is transient and is involved with the initial encoding of memory but not with its subsequent maintenance and storage.

Ouabain, in addition to, or as a consequence of its inhibiting effect on the cation pump, decreases ^{14}C-leucine uptake into chick brain synaptosomal fractions (Gibbs, Robertson and Hambley 1977), so it may be that amino acid transport driven by a sodium gradient occurs during the labile phase and is necessary for subsequent consolidation to LTM. This has been tested by injecting aminoisobutyrate (AIB), which is neither incorporated into protein or metabolized, but is transported across cell membranes, and in so doing should compete with other amino acids for transport sites, thus reducing their uptake. When given intracranially, AIB does indeed reduce ^{14}C-leucine uptake by the chick brain and consequently it might be expected to impair LTM by interfering with the necessary amino acid transport that is occurring during this phase. When it was given at different times after learning and retention measured at 180 minutes, memory was disrupted in a way that parallels the effects of ouabain (Gibbs and Ng 1977). Finally the effect of agents such as ouabain on labile memory can be distinguished from those of protein synthesis inhibitors such as cycloheximide on the basis of differences of time course and dose response; between 10 minutes and 90 minutes after learning, increasing the dose of ouabain increases memory decay whilst increasing the dose of cycloheximide is without effect over this time period (Gibbs and Ng 1977). This is consistent with the view that the consolidation of LTM takes longer than the time required for amino acid uptake, since aminoisobutyrate is effective only up to 10 minutes after learning (see below); the evidence for the involvement of protein synthesis will be discussed below.

1.6 Macromolecular synthesis

The remarkable resilience of LTM makes it highly probable that it is encoded by durable changes in cytoarchitecture. Any such structural 'rebuilding' would

invariably involve proteins, possibly synthesized de novo and maybe subject in addition to post-translational modification. So during the consolidation phase evidence for the mobilizing of the protein synthetic machinery of the brain would be expected, and indeed a vast amount of work has been addressed to this key issue. Two main approaches have been used; one has been to test the effect on learning of inhibitors of macromolecular synthesis, the other has attempted to measure the synthesis of macromolecules with precursor incorporation studies. From a biochemical viewpoint an important question is whether learning generates the production of unique species of macromolecules or only altered rates of turnover and/or net synthesis of normally constitutive material. In all these studies the difficulties of interpretation have stemmed as much from the behavioural as the biochemical aspects of the work, as will become apparent from what follows.

1.6.1 RNA inhibitor studies

Dingman and Sporn (1961) first showed that 8-azaguanine, an anti-metabolite of RNA which becomes incorporated into brain RNA when injected intracisternally, whilst it did not impair the ability of a rat to learn a water maze task, did produce amnesia for the task. On subsequent tests the injected animals made more errors than the controls. Such a result does not distinguish completely between an involvement of RNA synthesis specifically in learning or some secondary role on, say, motivation. In fact, evidence for an effect on motivation comes from a study with 8-azaguanine on operant conditioning (Jewett, Pirch and Norton 1965).

Actinomycin D is an antibiotic which inhibits DNA-dependent RNA synthesis, by binding to guanine residues in the DNA, and although its effects in mice are debatable, amnesia is produced in fish as a result of its intracranial injection (Agranoff et al 1967). The drug exerts gross toxic effects much later in these animals than in mammals and hence overt behavioural effects of illness which confounded the mouse studies are probably not present during the crucial training and testing period.

One interesting result was obtained with a study using the antimetabolite 2,6-diaminopurine intracisternally. This drug greatly increased the number of trials required to train rats on a dark avoidance conditioning task. The novelty lay in the fact that diaminopurine inhibition of RNA synthesis can be completely reversed by giving intracisternal adenosine. By varying the time of injection of this nucleotide it was possible to determine that RNA dependent learning processes seemed

to occur between 30 minutes and 75 minutes after the intial training (Allweis and Kobiler 1973). Other agents which inhibit RNA synthesis also affect retention. Thus camptothecin, a plant alkaloid which inhibits synthesis of high molecular weight nuclear, but not mitochondrial RNA, prevents retention of shuttle box learning in the goldfish (Neale, Klinger and Agranoff 1973) and 5-bromotubercidin, an adenine analogue, blocks both mRNA and rRNA synthesis and impairs memory, whereas 5-fluorouridine, which only inhibits the production of rRNA, is without behavioural effect (Agranoff 1974).

1.6.2 RNA synthesis studies

Precursor incorporation experiments conducted in this field relatively early on were subject to the criticism that the precursors were incorporated into the rapidly turning over heterologous nuclear ribonucleic acid (HnRNA) fraction (Dunn 1976), of unknown function, but which may consist of a large proportion of non-translated intron, and which is almost certainly not the direct precursor of mRNA. Likewise analyses of changes in base composition of RNA provided only inferential evidence for the synthesis of mRNA. Nonetheless it is worthwhile examing more recent claims which seem to circumvent these problems and which may clarify the nature of the nucleic acids involved.

Rats display preferential handedness when bar pressing for a food reward but will learn to reverse this handedness if constrained from using their preferred paw. Making use of the observation that the 3-terminal of mRNA contains polyadenylate residues which enable the RNA to be isolated using oligo (dt)-cellulose (Lim and Cannelakis 1970; Aviv and Leder 1972), Cupello and Hyden (1976a, 1976b, 1978) found that in hippocampal CA3 pyramidal neurons training reproducibly generated polyadenylate-associated RNA which migrated between 18S and 28S, but whether it was a nuclear precursor or a cytoplasmic nucleic acid was not known (Cupello and Hyden 1976a). The labelling of 8S and 16S RNA that occurs with training (Cupello and Hyden 1976b) has shown these species to be without polyadenylate residues, though they may still quite conceivably be mRNA's.

Interesting results have been found by Shashoua, using a task in which goldfish learn new swimming skills in response to having polystyrene floats fixed to their bellies; the floats turn the animals upside down and they have to learn to reverse their positions. In trained animals there is an enhanced synthesis of uracil-rich RNA from ^{14}C-orotic acid (Shashoua 1968). Sucrose density gradients of total

cerebral RNA showed that after a 4 hour pulse of labelled precursor, maximal incorporation occurred into a fraction sedimenting between 18S and 4S indicating that neither rRNA or tRNA was involved. After 24 hours, 28S, 18S and 4S fractions were all labelled; that is rRNA and tRNA were being synthesised.

It is disturbing however that fish given floats too large for them to be able to master the task in the 4 hour training period showed the greatest difference in U/C ratio. Shashoua argued that the fish were trying to learn and that stress was an improbable cause of the difference, since stress controls in which fish were forced to swim in a whirlpool produced no change (Shashoua 1970). However, forced swimming may not be equivalent in stress terms to being forced to remain upside down and no biochemical measures of stress were reported. Interestingly a correlation existed between the length of time a fish took to acquire the skill and the changed U/C ratio; the longer it took the higher the ratio. Could this possibly be because the longer the fish takes to right itself and swim naturally, the more stressful the experience?

This possibility cannot be ignored since in experiments involving shock avoidance conditioning in goldfish, shock alone resulted in an increased U/C ratio of the same order of magnitude as that found by Shashoua (Baskin, Masiarz and Agranoff 1972). Fish oxygen consumption can rise threefold during stress with a consequent rise in pCO_2 and fish subjected to an elevated CO_2 concentration showed an increased U/C ratio. This altered ratio was found in all cases to be due to decreased cytidylate labelling and it was postulated that the CO_2 raised cerebral blood flow, altering the steady state concentrations of glutamine which has been implicated as a cofactor for cytidylate synthetase.

The only technique which is currently sensitive enough to detect what might be small amounts of unique RNA is that of DNA-RNA hybridization and although a shock avoidance paradigm in rats did provide evidence for the synthesis of unique nucleic acid species (Machlus and Gaito 1969) but such experiments are no longer pursued, probably because they are relatively technically difficult to accomplish and grave difficulties in interpretation exist.

Given then that some evidence for increased mRNA synthesis in learning situations exists, are proteins also synthesized?

1.6.3 Polysomes

When mRNA is translated into protein on the ribosomes the latter (monosomes) become aggregated into multiple units called polysomes. An increase in the polysome/monosome ratio should therefore accompany enhanced protein synthesis.

Oddly CXM causes disruption of brain polyribosomes with a time course which indicates that it is independent of its effect on protein synthesis; that is, the polysome/monosome ratio returns to normal long before the drugs' inhibitory effect is lost (MacInnes and Luttges 1973).

Wenzel et al (1975) have reported an increase in the number of membrane bound polysomes, as determined by quantitative electron microscopy, in the rat hippocampus following the learning of a shock motivated brightness discrimination. At 75 minutes after training increased precursor incorporation into RNA and protein was found, and the number of bound ribosomes in both the CA1 and CA3 regions of the hippocampus was raised when compared with active controls. In the CA1 region training increased the number of free polysomes, but in CA3, polysomes were increased in both trained and active control groups compared with passive controls. While the response of the CA1 region then seemed restricted just to the learning situation, that of the CA3 region was a more generalized reaction. This apparent specialization of the CA1 cells does accord with their connections, which indicate that they comprise a later stage in information processing that CA3 cells.

1.6.4 Protein synthesis inhibitor studies

The initial choice of puromycin as an inhibitor of protein synthesis was an unfortunate one in some respects which created a degree of confusion because, as was eventually revealed, its effects in mammals (though not in fish) were brought about in an unexpected way. Originally it was apparent that to consistently lesion memory with puromycin it was necessary to inhibit protein synthesis by 80% for 8-10 hours (Flexner et al 1964). When mice trained on a conditioned avoidance task in a 'Y' maze to avoid an electric shock were given a regime of injections of the antibiotic one day after training into both temporal lobes, so as to achieve this amount of inhibition, memory was impaired. A reversal learning paradigm showed that the puromycin was not exerting its effect on performance (Flexner et al 1963). Strangely though memory could still be lesioned up to 43 days after training by injecting the drug widely into the neocortex and thalamus, the transition

from memory affected by bitemporally injected puromycin to the generalized effect seem to occur between 3-6 days. Secondly, intracerebral saline could restore puromycin lesioned memory even after two months (Flexner and Flexner 1967). Puromycin has a structure similar to the amino-acyl end of tRNA and binds to the carboxylate groups of nascent polypeptides causing their premature termination and release from the ribosome. It was thought that these small, abnormal peptides might be toxic and interfere with some process required for memory, and that the restoration following saline was due to the Na^+ ions 'inactivating' these toxic molecules. Consistent with this, puromycin does not affect memory when administered together with acetoxycycloheximide (ACXM), which inhibits the formation of peptidyl puromycin by preventing the transfer of amino acids from tRNA to the growing chain (Flexner and Flexner 1966). The idea was further supported by a study of instrumental conditioning in quail (Mayor 1969) and by finding peptidyl puromycin with paper chromatography of trichloracetic acid (TCA) precipitable material as long as 58 days after treatment with the antibiotic (Flexner and Flexner 1968). All of this suggests that the peptidyl-puromycin is preventing recall.

Using an active avoidance task in which mice were trained to avoid electric shock by choosing the lighted limb of a 'T' maze, Barondes and Cohen (1968) found that ACXM inhibition of protein synthesis impaired retention between 3-6 hours after training, but that the protein synthesis necessary for LTM took place within a short time of the training. Injection 5 minutes after training produced a much reduced amnesia. So whilst the proteins required for LTM are generated soon after training, a STM, independent of protein synthesis lasted from 3-6 hours. That synthesis early on is involved is further supported by the impaired learning that seems to occur about 40 minutes after injection with cycloheximide (CXM), but animals also become hypoactive at this time. However treatment with an analogue, isoCXM, which has similar effects on motor activity did not impair learning (Squire and Barondes 1973), and doses of amphetamine that antagonise the antibiotics' amnestic action does not alter its effect on activity (Segal, Squire and Barondes 1971).

Spontaneous return of memory 6 days after training has been observed in rats treated with ACXM (Serota 1971). The long time interval suggested that it was recall, rather than consolidation that was being deranged. Re-exposure of the animal to the training apparatus was sufficient to revoke the amnesia, and this recovery could itself be attenuated by CXM (Quatermain, McEwen and Azmitia

1972). That this is not a recall deficit has been argued on the grounds that it occurs only when the drug is administered before the training but not after. Surely a retrieval mechanism operative days later would not be dependent on events occurring in the first few minutes around the time of training. An alternative was that a third phase of memory independent of, and occurring after, the CXM sensitive period permits recovery (Squire and Barondes 1972). Other studies which could not demonstrate such recovery, have cast doubt on the training method of Squire and Barondes (Flood et al 1973), and ruled out retrieval deficits (Barondes and Cohen 1968; Mark and Watts 1971).

One particularly elegant experiment set out to demonstrate whether learning is dependent on normal constitutive proteins with short half lives (Squire and Barondes 1976). A comparison between animals trained either with 90% inhibition established by CXM one minute before, with those inhibited 118 minutes before training revealed that a hypothetical constitutive protein with t/2 = 10 minutes could not be involved in learning. Mice injected one or 5 minutes before training, in whom depletion of such a constitutive protein would be minimal, exhibited impaired retention; but those injected 118 minutes prior to training, in whom a considerable turnover of constitutive protein could be expected, showed no impairment. Incidentally this experimental design ruled out the possibility that abnormal amino acids produced as a result of drug effects, e.g. elevation of tyrosine by CXM, generated the amnesia. If this were the case amnesia should have been greater in the 118 minute group. The same would also be true if the effects of CXM and ANM (anisomycin) on tyrosine hydroxylase and of ACXM on catecholamine synthesis, which are slow to develop, were on memory (as has been proposed, Flexner and Goodman 1975). Given the largely inferential nature of conclusions that can usually be drawn from the use of inhibitors, because of problems about uncertainty of the mode of action, non-specificity of behavioural effects and toxicity, this study highlights just how informative such experiments can be if designed to answer appropriate questions.

In young chicks intracranial CXM permanently impairs subsequent learning acquisition. The onset of the period of greatest sensitivity to CXM on visual learning tasks coincides with the onset of the sensitive period for imprinting, i.e. 18-24 hours; whereas sensitivity of auditory learning to CXM occurs as early as 6 hours, when it is known that birds will imprint on sounds (Gottlieb and Klopfer 1962). Modality specific protection against this CXM induced impairment has been

demonstrated. Keeping birds in darkness or in sound free environs prevents visual or auditory learning deficits respectively, but not the converse (Rogers, Drennen and Mark 1974). The learning lesion then reflects some action of the drug on the development of mechanisms by which sensory input is processed. Moreoever the visual input which accompanied the CXM generated learning deficits in young chicks had to include intersecting lines or spots presented as the centre of the frontal field of vision. Patterns in the peripheral visual field were without effect. Ganglion cells in the chick will respond to edges but not to angles (Miles 1972) so modification by the drug of processing at the retinal level seems unlikely. Probably it is the hyperstriatum, with its organization similar to the mammalian visual cortex (Revzin 1969) which is sensitive to the drug at what must be a critical stage in development (Rogers and Drennen 1978).

Even the simplest kind of learning, habituation (Squire and Becker 1975) is prevented by anisomycin (ANM). Further ANM exerts a behavioural effect which correlates well with dosage (Squire and Davis 1975) and with duration of action (Flood et al 1973). These antibiotics exert their effects by distinct means. The glutarimides such as CXM inhibit peptide bond formation, whereas ANM blocks translocation at the ribosome but does not directly interfere with peptide bonding. They generate different side effects, and these have frequently been demonstrated to be dissociable from their amnestic properties. Three different classes of antibiotic, then, represented by puromycin, CXM, and ANM all interfere with LTM, and on the basis of these inhibitor studies it seems reasonable to postulate that protein synthesis is necessary for consolidation of LTM.

1.6.5 Alterations in proteins

A number of studies have shown that incorporation of amino acids into protein fractions is changed by a training experience, one interpretation of which is that the rate of turnover of proteins may be a correlate of learning. Evidence for the involvement of proteins in the consolidation of LTM have also been adduced by studying alterations in identified proteins or the effects upon training of antibodies raised against either protein fractions or purified proteins.

One fundamental question is whether what is synthesized in response to training are normal constitutive or unique proteins. A decade or so ago a modicum of support was given to a model in which the synthesis of unique 'information containing' macromolecules coded in some undefined way certain neural 'circuits' which resulted in learning. It was a school of thought errected on and having as its hallmark the

so called 'transfer of learning' paradigm, and so fraught with difficulties at behavioural and biochemical levels were these experiments that justifiable interpretations were seldom possible (Rose and Longstaff 1983). Increasing knowledge about the effects of peptide hormones on stress, motivation, learning etc. may in the future permit a re-evaluation of these data in somewhat different terms and at present it seems best to regard the synthesis of unique proteins as, at the very least, unproven.

The work of Hyden's group is noteworthy in that they have consistently used the same transfer of handedness paradigm for well over a decade and have developed elegant microtechniques which enable processing of nerve cells individually dissected from a block of tissue. Hyden and Lange (1968) have shown that the incorporation of ^{3}H-leucine into hippocampal CA3 region pyramidal neurones is increased in a group of rats trained for 4 to 5 days to use their non-preferred paw to obtain food. The authors corrected their incorporation data for fluctuations in ^{3}H-leucine concentration (i.e. relative specific activity) and the uncorrected specific activity is considerably less in the experimental rats than the controls which learn the identical operant conditioning task, but with the preferred paw. Two problems are immediately manifest here. Firstly considerable difference in precursor availability exist between experimental and control animals, and secondly both groups learn.

An attempt to explore the behavioural significance of the earlier findings was more impressive with regard to the second point. This investigated the incorporation of radiolabelled valine into 2 groups of rats bred for their differential ability on a two way avoidance task; one group is bright, the other dull. Although finding that the incorporation of valine is elevated by training only in the bright rats (Hyden Lange and Seyfried 1973) it is a fairly generalized effect occurring in visual and sensory motor cortex as well as two limbic areas, and the authors do not report whether the rats are bright and dull in respect of the reversal of handedness task. Perceptual or motor defects in the dull group are not excluded.

Later studies (Hyden and Lange 1976; Hyden Lange and Perrin 1977) have shown a bewildering number of changes of incorporation of ^{14}C-valine into proteins separated on polyacrylamide gels. At 24 hours after training incorporation is elevated into a band represented in gels of hippocampus, thalamus and visual cortex, though the resolution of the gels appeared poor. After four days of training there is a transiently increased incorporation into two hippocampal synaptosomal fractions of

30,000 and 80,000 daltons, and in cortical synaptic membranes incorporation is elevated into an increasing number of fractions as training proceeds. At 19 days there are further cortical cell synaptic membrane increases in turnover and total quantity of 39,000, 49,000, and 90-110,000 dalton protein fractions.

Later still, at 36 days cortical cell synaptic membranes show increased incorporration into proteins over virtually the whole range of molecular weights between 30-70,000. One of the difficulties in interpreting just what these changes mean is the complex training schedules used, so the picture built up is not a true time course but possibly confounded by any number of training artifacts. Systematically attempting to characterize and ascribe physiological roles to these proteins would be a daunting task.

One of the proteins identified by polyacrylamide gel electrophoresis as having increased in total quantity by 5 days of training is the brain specific S100 protein in hippocampal CA3 neurones (Hyden and Lange 1968). Also taken as evidence for the importance of this protein in learning is the impairment produced by the intraventricular injection of anti-S100 serum. Similar administration of the serum adsorbed onto S100 protein, or anti-γ globulin serum was without effect on subsequent training. Immunofluorescence demonstrated the presence of S100 antibody in hippocampus, but not in the brain stem of those animals which had received serum unabsorbed by its antigen (Hyden and Lange 1970). Further work revealed that whilst control animals showed a single S100 band on PAGE, trained rats showed two, which was thought to be due to the appearance of a second conformation of the protein, Next, training increased the Ca^{2+} content of the CA3 region and since Na^{+} and K^{+} content did not alter, the divalent ion concentration change was intracellular rather than intravascular. This is interesting because S100 is stabilized in its native conformer by Ca^{2+}, as indicated by the protection the ion affords against thermal denaturation (Kessler et al 1968). Two possibilities were either that the synthesis of a new conformer was stabilized by calcium ions or that an increase in the calcium ion levels altered the conformation of originally homologous S100 protein. No stoichiometric relationship existed between the increase in S100 and Ca^{2+}; far more calcium being generated than necessary to stabilize a new conformer, which seemed to support the latter hypothesis (Haljamae and Lange 1971).

Other workers have found alterations in S100 after visual stimulation (Singh and Talwar 1969) and after learning a 'T' maze task (Zomzely-Neurath et al 1977).

Impairment to maze learning has also been seen after subdural injections (which do not damage the brain or blood-brain barrier as do intracranial injections) of an anti-S100 serum and an anti-serum raised against synaptic plasma membranes (SPM). It is worth noting that this study found that whilst anti-SPM produced recurrent spiking activity, anti-S100 did not (Karpiak 1976); implying that different roles are played by the two antigens. In this connection it is noteworthy that anti-SPM impairs learning in a wide variety of paradigms, without effect on acquisition, motivation or motor activity which is evidence for a specific lesion of recall mechanisms (Kobiler et al 1976). Such processes seem to be sensitive to antibody when given between 24-72 hours after training, which presumably represents the phase of learning dependent on the target antigens.

However it is worth considering that the immunologic interpretations may be simplistic for the assumption is generally made that antibodies lesion memory directly through their effect on target antigen, but this need not be the case. In combining with an antigen which is an integral part of, for instance, a synaptic plasma membrane, it might be that conformational changes imposed on neighbouring though antigenically unrelated, molecules could account for the derangement of learning.

A somewhat simpler pattern of changes in protein metabolism than those found by Hyden accompanies the acquisition of a difficult swimming skill by goldfish (see 1.6.2). Using a pulse of radiolabelled valine it has been possible to identify 3 cytosol proteins by PAGE, which have enhanced incorporation, termed α, β and γ with molecular weights respectively of 37, 32, and 26,000 daltons. Immunological studies suggest that β and γ represent single antigens normally present in goldfish brain (Shashoua 1977).

The experimental design which Shashoua uses may not eliminate stress as a cause for these changes. Fish which learn the task rapidly do not show the alterations in incorporation, maybe because they are the least stressed. However it is worth noting that cortisol levels are not enhanced to the same degree during the learning task as during the whirlpool stress control (Kaplin and Sirlin 1975). Arguing in favour of a learning specificity is that the changes do not occur in animals which do not learn or animals that acquire the skill and 'forget' it later. This latter evidence says that the differences are not acquisition, but storage related.

An immunohistochemical study of the localization of the β protein revealed that it is associated with ependymal tissue, mostly close to the III ventricle of diencephalon, optic ventricle of the mesencephalon and the vagal lobes of the hindbrain. The protein then is almost certainly restricted to non-neuronal cells, and it is difficult to imagine how such could respond plastically to acquisition of a vestibulomotor skill (Benowitz and Shashoua 1978).

The subsequent injection of anti-β or anti-β plus anti-γ had no influence on acquisition, but did affect recall when given from 3-48 hours, but not 72 hours after training (Shashoua and Moore 1978), but it is difficult to see how antibodies could react against soluble cytoplasmic proteins to produce this amnesia. The time course suggested that the proteins were not important for acquisition of final encoding but needed only for consolidation. More recently however the β and γ proteins have also been identified in the ECF and CSF so the argument now is that these proteins are secreted into the ECF, whereupon they migrate to target sites (Shashoua 1979). This overcomes the difficulty in understanding how the antibodies exert their amnestic action.

While the work of Shashoua posits a rather unusual mechanism, namely the synthesis of a learning modulator distant from its presumed site of action, recent work in the chick seems to extend the somewhat more conventional notions about the role of protein synthesis with training. One trial passive avoidance learning results in increased incorporation of ^{14}C-leucine into TCA precipitable material from the anterior dorsal forebrain at 30 minutes after training into both a soluble and particulate fraction. The incorporation remains elevated in the soluble fraction at 24 hours, but by 48 hours neither fraction has an incorporation significantly different from control values. The increased labelling of the particulate fraction was not apparently due to incorporation into synaptic membrane tubulin (Mileusnic, Rose and Tillson 1980).

If, as current dogma would assert, learning is mediated by alterations in connectivity, then structural and functional changes at the synapse presumably require that proteins synthesized de novo are exported from the cell body down the axon, and in this respect microtubules seem important. Indeed a number of indirect pointers attest to a possible role for microtubule protein in plastic adaptations associated with light exposure (Cronly-Dillon and Perry 1976; Rose, Sinha and Jones-Lecointe 1976; Stewart and Rose 1978; Potemska and Rose 1980). Rather more specifically

colchicine, which binds to the tubulin dimer, seems to impair learning in goldfish (Clingbine 1978) and chicks. In the latter species two experiments are noteworthy. In the first, three groups of birds were used in a one trial passive avoidance study (see 1.5). A control group previously injected with saline pericardially was presented with a red lure coated with water. A second group which had also been injected with saline were presented with a red lure tainted with MeA, whilst a third group though also presented with the tainted lure had been injected with colchicine. The birds were subsequently tested by being presented separately with a red and a blue lure. When the total number of pecks given to each lure during the test was considered it was evident that colchicine appeared to depress total pecks which may indicate an effect of the drug on arousal. When the results were expressed as median peck suppression (where peck suppression for each bird was the number of pecks to the blue lure as a percentage of the total pecks to both lures) it was apparent that the colchicine treated group was not significantly different from the control group in terms of the number of pecks made to the red lure. By contrast the saline injected aversively trained group had significantly fewer pecks to red than either of the other groups. The implication was clear; the alkaloid had impaired avoidance, at least in part. Larger doses of the drug may have proved to be completely amnestic but the authors had chosen the dosage to minimize effects on activity. The study did not reveal whether the drug acted on acquisition, storage or recall, and altered arousal could not be ruled out (Cherfas and Bateson 1978).

The second experiment injected chicks intraperitoneally either with colchicine or saline prior to training on an imprinting stimulus. Colchicine injected birds suffered impaired retention on the following day (Bateson and Rose, unpublished data) but the drug also increased distress calling during the test when compared with the saline controls and so an effect on performance could not be ruled out.

Generally protein synthesis does seem to accompany learning and two proteins in particular which may be important, namely S100 and tubulin, have been identified, although functional roles, especially for the former can as yet only be guessed at.

1.6.6 Post-translation modification

Once synthesized, proteins may be further modified by, for example, glycosylation or phosphorylation. Indeed it is quite conceivable that these alterations may occur not only to proteins generated in response to learning but also to pre-existing molecules. Currently no attempt has been made to distinguish between these possibilities,

but some progess has been made towards implicating the importance of post-translational modulations generally in learning.

Several studies seem to militate against a role for glycoproteins in learning. Holian, Brunngraber and Routtenberg (1971) found no differences in (1-^{14}C)-glucosamine incorporation between rats trained on a one trial passive avoidance of footshock task and shocked or unshocked controls. Moreover no changes were noted in amounts of N-acetylneuraminic acid (NANA), hexose, hexosamine or fucose. Further 1 hour after escape training no differences in activity of galactosyl or N-acetylgalactosaminyl transferases were seen (Zingarelli, Mallory and Raghupathy 1975).

Using an operant conditioning task in pigeons, Bogoch (1970, 1974) was able to demonstrate that a particular glycoprotein fraction separated on Cellex D was present to a greater extent in birds which had learned best compared with those that were poor learners. Two of these fractions designated 10B and 11A had similar amino acid compositions but quite different carbohydrate composition. Since the concentration of 10B was high early on after the training and later declined to pre-training levels it was suggested that training involved a transition from 10B to 11A, and that this would involve altering the heteropolysaccharide residues of the glycoprotein, which in its turn brings about interneuronal recognition. Another study, that of Barraco and Irwin (1976) claimed to have identified the same protein fractions as Bogoch but which in their birds were present in reduced amounts in both learning and active control groups compared to passive controls, which do not key peck at any time. Additionally changes were found in amounts of one soluble and one insoluble (100,000 daltons) protein in the birds which acquired a new key peck task compared with those which were overtrained. This study failed to show any subcellular localization for, or anatomical differences in, the incorporation of either ^{3}H-valine or ^{14}C-glucosamine (Irwin, Barraco and Terrian 1978), which is curious.

Despite this work evidence for the involvement of altered glycoprotein metabolism in response to both light stimulation and learning exists. Shock motivated brightness discrimination increased the incorporation of intraventricularly administered (1-^{3}H)-L-fucose into high molecular weight glycoproteins of CA1 and CA3 hippocampal neurones by comparison with active and passive controls. No change was

seen in the visual cortex. Routtenberg et al (1974) finds an increased incorporation of ^{3}H-fucose into low molecular weight glycoproteins of a caudate nucleus crude synaptosomal fraction of rats trained on an active avoidance task. The caudate is part of a dopaminergic 'reward' system in the brain which has been implicated in learning processes. Decreases in incorporation in the shock control group may be the result of stress, since sham intravenous or intracranial injections, which presumably involve more than the usual degree of restraint, attenuates the increased incorporation of ^{3}H-fucose that is contingent on environmental stimulation (Damstra et al 1975). An alternative explanation pointed out by the last authors, is that there are likely to be dissimilar kinetics for various routes of administration. Interestingly an increase of ^{3}H-fucose incorporation into TCA precipitable material of a particulate fraction from the anterior dorsal forebrain of neonatal chicks was detectable 30 minutes, 3 and 24 hours but not 48 hours after one trial passive avoidance learning (cf 1.5) indicates that learning may be accompanied by increased synthesis of glycoproteins de novo or fucosylation of pre-existing proteins. Between 24 and 48 hours, although incorporation had continued to rise in both the MeA (trained) and control groups, the specific radioactivities of the two groups had approached similar levels by 48 hours, hinting that glycoproteins produced as a result of training had fairly short half-lifes compared with the constitutive glycoproteins which were presumably monitored by the control group. (Sukumar, Rose and Burgoyne 1980).

It is usually presumed that glycoproteins synthesized in the neuronal perikaryon are transported to the synapse where they effect structural and/or metabolic modifications but is worth noting that there is both in vivo (Barondes 1968) and in vitro (Dutton, Haywood and Barondes 1973) evidence for glycosylation at the synapse, presumably mediated by synaptosomal mitochondria, with which glycosyltransferases are known to be associated (Bosman 1971). Specifically however, fucose incorporation appears not to be synaptosomal (Zatz and Barondes 1971).

Passive avoidance of footshock training in rats caused a transient increase in the incorporation of labelled orthophosphate, given intracranially via the optic foramen, into phosphoserine of nuclear non-histone proteins. Incorporation into monophosphate nucleotides did not alter. In accordance with this the molar ratios of phorphoserine and serine increased with training to the same extent as would be

predicted by the tracer labelling study. This increased phosphorylation (or decreased dephosphorylation) seems to be localized to limbic structures such as the amygdala, entorhinal cortex, hypothalamus or hippocampus (Machlus, Wilson and Glassman 1974) and is not the result of motor activity or handling. It will occur in rats that have been trained, extinguished and then either retrained, reintroduced to the experimental apparatus or simply handled. What is suggested by this is that the avoidance task produces some permanent brain change which, when triggered by appropriate clues, produces the alterations in phosphorylation. That the trigger requires pairing of unconditioned and conditioned stimuli and does not just reflect increased emotionality seems reasonable, as randomly shocked animals show no such changes. It is difficult though to see how handling alone acts as it does, since we are told that all the animals are gentled by handling for two days before the start of the experiment, therefore handling would not be expected to be paired with the experimental set up. If the authors interpretation is right then one could view the increased phosphorylation as a concomitant of recall rather than storage, and which is triggered by 'reminder' cues in the training situation.

Phosphorylation was increased in synaptosomal particulate fractions of footshock avoidance conditioned mice compared with quiet controls. This was not due to changed AMP pools. Extensive characterization of the phosphorylated material demonstrated that it consisted of more than two phosphoproteins. It was impossible to determine whether the incorporation consisted of synthesis of phosphoproteins or decreased phosphorylase activity, or whether the effect was pre- or post-synaptically located (Gispen et al 1977). Various behavioural controls showed that the phosphorylation was not only training specific, but also occurred with extinction of the conditioning (Perumal et al 1977).

Having considered certain biochemical substrates which may underlie both STM and the establishing of LTM it is necessary to consider whether consolidation can be modulated and if so, how?

1.7. Arousal and consolidation

Squire and Barondes (1976) came to the conclusion that the memory impairment by the glutarimide antibiotics was due to their ability to inhibit the synthesis of proteins and not an action on catecholamine metabolism as had been claimed (Flexner and Goodman 1975). This was supported by the finding that a competitive inhibitor of tyrosine hydroxylase, α-methylpara-tyrosine, does not affect memory

fixation (Squire, Kuczenski and Barondes 1974).

However, a number of reports are extant in the literature which suggest that things are not that simple. The first hint that all was not well came with the realisation that overtrained mice were not affected by puromycin (Flexner and Flexner 1967), maybe because, it was suggested, over a prolonged training period even the low level of protein synthesis remaining was sufficient to 'encode' the memory into long term store (Barondes and Cohen 1967). However as we have seen, in mammals, problems arise because of the formation of toxic metabolites. An alternative explanation, supported by experiments with an active avoidance task in goldfish (in which species puromycin is not toxic) proposes that the time of fixation into long term store can be protracted by high levels of arousal, which is maintained by keeping the animal in its training environs during overtraining (Springer and Agranoff 1976). What is effectively happening with both the mice and the fish is that the heightened arousal maintains the short term processes so that the 'fix memory' signal (to use Agranoff's term) and hence the consolidation into LTM does not occur until after the protein synthesis inhibiting effects of the antibiotic have worn off.

That the level of arousal should determine the time at which consolidation occurs may help to explain a number of paradoxical results and also serve to highlight the importance of behavioural variables which superficially may seem trivial. For example, it has been noted that amnesia produced by CXM for a one trial learning paradigm in mice could be counteracted by individual as opposed to group housing of the animals (Golub et al 1970) as social isolation increases arousal. With a similar paradigm in the same species Flood et al (1972) found that overtrained animals would not show evidence of amnesia as a result of inhibition of protein synthesis until the training to testing interval was extended.

Kety has expressed the view that steroid hormones released as a result of stress, anxiety and heightened arousal that accompany learning acted to protract the time course of consolidation (Kety 1972). However, several hours are required for steroids to exert their cellular response so are unlikely to be important in the short term. An alternative thesis also proposed by Kety has it that catecholamine levels are a correlate of the affective state of an animal and that via a large number of unspecific aminergic afferents projecting to the cerebral cortex, the response of specific connections mediating learning are modulated to enhance

acquisition and accelerate consolidation. This means that sympatholytic drugs should retard consolidation, but doubt has been cast on whether these agents have any effect on learning (Squire and Barondes 1976), and even if they do it is said to be on recall. However, if instead it is postulated that since the amnesias produced by protein synthesis inhibitors can be countered by the sympathomimetics, imipramine, amphetamines and MAO inhibitors such as tranylcypromine (Roberts, Flexner and Flexner 1970; Serota, Roberts and Flexner 1972; Botwinick and Quatermain 1974), these drugs might act in a similar way to arousal, in that they prolong consolidation, as postulated by Agranoff.

There is other evidence for a noradrenergic modulation of learning. Reserpine, which depletes brain amine stores, if injected immediately after training impairs acquisition. When given either 30 minutes before or after training diethyldithiocarbamate (DDC), which blocks noradrenalin (NA) synthesis by inhibiting dopamine-β-hydroxylase, prevents the expression of memory for a one trial passive avoidance task 24 hours later in mice (Randt et al 1971). In the rat a similar blockade can be prevented by the intraventricular administration of NA within an hour of the training trial (Stein, Belluzi and Wise 1975).

Against this view would appear to be fascinating evidence from work in which forebrain NA is almost totally depleted by the injection of young rats with 6-hydroxydopamine (6-OHDA). One study found that such treatment had no effect on acquisition of an appetitively conditioned task, though it did produce a perseveration of extinction (Mason and Iversen 1975). Whilst these authors were correct in asserting that this lends no support for NA being necessary for learning it does not undermine the original contention of Kety that the affective state may <u>influence</u> learning.

However, when Mason and Fibiger (1979) investigated the effect of stereotactic injection of 6-OHDA into the dorsal bundle of rats, they discovered that the treated rats acquired the task more rapidly than the controls, probably because they had a shorter freezing response which facilitated movement between the two halves of the shuttle box. This is consistent with the notion that the dorsal noradrenergic bundle is involved in fear/anxiety. This is not a trivial point since one study found that bilateral lesions of the locus coeruleus, which give origin to the A6 fibres of the dorsal noradrenergic bundle, diminished the rate of running in rats learning a maze for a food reward (Anzelark et al 1973). Moreover DDC, reduced self-

stimulation rates in rats, an effect which could be reversed by intraventricular injections of NA (Wise and Stein 1969). These findings might at face value indicate that the lesion or drug was damaging the reward system of the brain and not arousal. It has been argued that the reward system is noradrenergic (Wise and Stein 1969) and if this is the case then sympathomimetic drugs may be acting on reward rather than arousal. Although self-stimulation does occur with electrodes placed adjacent to the locus coeruleus, the close proximity of the dopaminergic A9 and A10 fibres with this nucleus, which gives origin to the A6 noradrenergic tract (Dahlstrom and Fuxe 1964) would seem to make the matter of which neurotransmitter is involved far from clear. However the dopamine antagonist spiroperidol reduces self-stimulation rates in rats markedly, without any effect on the indices of arousal measured (spontaneous rearing and locomotor activity), indicating that the reward system is dopaminergic. This was also the conclusion advanced in more recent comprehensive reviews of the catecholaminergic theory of reward (Wise 1978; Fibiger 1979) the latter of which also stated that noradrenergic pathways were almost certainly not involved. Moreover Roll (1974) was convinced that the locus coeruleus lesion, which reduced running in rats learning a maze, resulted in an arousal rather than a learning deficit.

To recapitulate: Arousal may protract the time over which consolidation to LTM takes place. There is pharmacological evidence suggesting that noradrenergic mechanisms are involved in learning and that these are most likely to be related to arousal rather than reward. It is worth recalling at this point however that arousal levels may be determined by reward (cf 1.3)

It can now be appreciated that the significance of any external event about which it is appropriate for an animal to learn is derived through the reward system of the brain and expressed as a particular degree of arousal. Highly significant events are highly arousing, and are therefore more readily learnt. Further, consoliation into LTM being protracted by high arousal levels might permit the maximum amount of information to be gained and processed at STM level; maybe leading to an LTM which is more accurate, more easily recalled or less prone to forgetting.

The arguments about the way in which arousal might modulate consolidation can now be extended to the molecular level.

Gibbs and Ng (1977) have argued that sympathomimetic drugs such as diphenyl-

hydantoin (DPH), amphetamine and NA counteract the amnesia produced by sodium pump inhibitors. It is known that noradrenergic neurotransmission is mediated by a cyclic 3':5' adenosine monophosphate (cAMP) system (Nathanson 1977; Greengard 1979). cAMP will stimulate amino acid uptake by chick forebrain slices (Hambley, unpublished experiments). Far more interestingly AIB uptake into the dorsal forebrain and midbrain of chicks was increased after one hour of training on an imprinting task, which was exactly the same time as there was an elevation in cAMP levels compared to light and dark controls (Hambley and Rose 1977). Since AIB is non-metabolizable the altered uptake must have been due to either change in transport mechanisms or blood flow. That the latter is not needed for such an effect is shown by the in vitro experiment.

Although the chick imprinting cAMP study was originally undertaken on the rationale that events generated by the imprinting might trigger the alterations in RNA metabolism via this second messenger, the evidence suggested otherwise. Such a model would predict increases both in adenyl cyclase, then cAMP, occurring earlier than the changes in RNA metabolism. However, cAMP levels in the dorsal forebrain of imprinted birds, after 15 minutes exposure, were lower than either light or dark controls and became increasingly higher than these controls only after 30 and 60 minutes exposure. Certainly after 60 minutes exposure there is an increase in maximally (fluoride) stimulated adenylate cyclase activity in the dorsal forebrain which suggests a rise in the total quantity of enzyme, but its relatively late appearance (RNA polymerase changes were seen 30 minutes after imprinting) is further evidence against the model originally proposed. Moreover, dibutyryl cAMP was without effect on in vitro RNA polymerase activity (Hambley and Haywood, unpublished work).

It can be argued that it is the labile phase of memory which is protracted by the greater noradrenergic stimulation that represents increased arousal. It might then be postulated that DDC lowers the stimulation of the cation pump by endogenous NA to a point where amino acid transport is sufficiently impaired to damage consolidation. Admittedly this does contradict the findings of Mason and Iversen (1975) though it may be that with the chronic depletion of forebrain NA in their experiments the cation pump develops a higher non-stimulated activity, which is capable of supporting a sufficient rate of amino acid transport for learning to occur. It is also known that if the pump is poisoned, or if the transport mechanism is saturated by non-metabolizable amino acids, such as AIB, that consolidation does not occur (Gibbs, Robertson and Hambley 1977). It could be inferred from all this that the

'fix memory' signal (to use Agranoff's term) which initiates consolidation, does not occur until the sodium pump generated amino acid uptake falls off, and further, that if the pump activity is depressed below a critical level, or maybe the rate of change of pump activity is particularly high, consolidation does not occur at all.

It is difficult to see why certain metabolizable amino acids such as proline or glutamine (Van Harreveld and Fifkova 1974) given intracranially also impair memory; possibly because here too saturation of transport sites prevents the normal rate of uptake of other 'essential' amino acids involved in this phase of memory. In support of this, the uptake of ^{14}C-leucine by forebrain is reduced by AIB, although the transport of other classes of amino acids seem to be differently affected (Gibbs, Robertson and Hambley 1977). Because of this, despite the absence of a global change in protein synthesis produced by the AIB, it is possible that distortions in amino acid availability would occur which derange the synthesis of a relatively small population of proteins generated in response to sensory stimulation.

L-proline, which results in heavy labelling of the hippocampus when injected into chicks, is a more powerful amnestic agent than either its D-enantiomer or a homologue, suggestive of a highly specific stereochemical requirements for whatever process is involved (Cherkin and Van Harreveld 1978). It may be interesting in this connection that L-isoleucine has no amnestic effects (Van Harreveld and Fifkova 1974). Certainly the explanation given by these latter authors, that these amnestic amino acids interfere with glutamate-induced swelling of dendrites which represents STM cannot be the case, as glutamate is itself amnestic (Gibbs and Ng 1977).

Specific predictions which emerge from this noradrenergic hypothesis of arousal which to my knowledge have not been tested are:- (I) Sympathomimetic drugs should stimulate the uptake of amino acids by chick forebrain both <u>in vitro</u> and <u>in vivo</u>. (II) Altering the state of arousal of chicks it should be possible to vary the time course of amino acid uptake by the chick forebrain. In practice this might be difficult to do, though maybe social versus isolated housing might succeed. The converse of this experiment would be to examine the time course of amino acid transport in both hemispheres of a 'split-brain' chick, with one side trained and the other naive, but where both sides would be similarly aroused. The hypothesis predicts that even though only one side of the brain has learnt, amino acid trans-

port kinetics will be the same on both sides because it is arousal and not learning related. (III) If NA is the modulator of consolidation timing then the differences brought about by social versus isolated housing would not be expected in animals depleted of forebrain NA by 6-OHDA. In this regard however the short term memory phase of Gibbs and Ng (1977) which is not cation pump dependent may also be protracted by isolating housing, hinting that several mechanisms may be responsible, at different times, for the overall effect.

1.8 Synaptic connectivity

Any neurochemical model of learning must explain how the sorts of process previously described results in the long lasting modifications in synaptic connectivity. Indeed plasticity of any type might be expressed - if a prime 'article of faith' for most neurobiologists holds true - by modifications at the synaptic level; as the growth of new dendrites, by neuronal cell death, or the selective gating of particular synapses, all of which might be said to alter the 'circuitry' of neural pathways. Although there is no paucity of possible mechanisms (see e.g. Kosower 1972; Rahmann, Rosner and Breer 1976), robust evidence for any in particular is hard to acquire. Whilst a considerable amount of work has implicated morphological changes at the synaptic level, for example in dendritic branching (Valverde 1967, 1971), synaptic dimensions (Cragg 1967) and numbers of synaptic vesicles (Vrensen and deGroot 1974, 1975), most of it has been concerned with rather general experiences such as rearing in enriched or improverished environments, which of necessity can reveal little about learning. I will concentrate then on rather better defined examples.

The most obvious substrate for change at the synapse would be in neurotransmitter metabolism, uptake, release, or in receptor mechanisms; none of which need be mutually exclusive. It is worth mentioning that the first three could conceivably involve glial cells which must be the most neglected aspect in this field of research. Such broad statements ignore the fact that there is no certainty about which neurotransmitters are involved in, for example, the hippocampus, amygdala, cerebral cortex and hyperstriatum (of birds) which have been implicated in learning. Nevertheless for one neurotransmitter system at least, evidence for involvement is now forthcoming.

The most comprehensive studies have been those of Deutsch and colleagues, using inhibitor drugs. Rats were trained to go to the illuminated arm of a 'Y' maze to avoid electric footshock. At various times after training the acetylcholinesterase (AChE) inhibitor, di-isopropylfluorophosphate (DFP), was injected into the hippocampus and retention was tested 24 hours later. Over the course of the first day, the older the memory the less it was affected by the anti-cholinesterase. After three days however, reversal occurred and by 14 days there was complete amnesia, which was not due to normal forgetting since controls showed almost perfect retention over the period. Similar results were obtained using an appetitive rather than an aversive task (Wiener and Deutsch 1968). However, such experiments do not eliminate the effect of a drug on performance of the retraining task than on recall processes. By contrast to the inhibitors of AChE, anti-cholinergic drugs such as scopolamine cause the greatest amount of amnesia between 1 and 3 days after training (Deutsch and Rocklin 1967).

If learning is dependent on the level of cholinergic transmission at particular synapses then increasing the level of transmitter using sub-maximal doses of anticholinesterase should facilitate recall. This does occur with undertrained animals (Deutsch and Rocklin 1967; Squire 1970), but the reverse is true for well-trained animals because they already show increased cholinergic transmission in response to learning and the additional drug produces a blockage. It was found for instance that DFP facilitated retention of the task after 28 days when control animals remembered it only poorly, whilst at 14 days when control rats showed good recall the experimentals suffered impaired retention.

A corollary to this would be that extinction might involve weakening of previously established connections or the growth of new connections that oppose those set up by the original learning. Rats trained, extinguished after 4 days, then injected with physostigmine, an inhibitor of AChE, and tested after a further 3 days should have an amnesia for the original training 7 days previously, but not for the extinction of training. The fact that such rats took twice as long to retrain when compared with controls that had been trained and extinguished 7 days prior to injection, indicated that extinction was a separate process from the original training. This favours the view that extinction may involve new synaptic connections (Deutsch 1974).

A further complication to the analysis of the involvement of cholinergic mechanisms in learning and memory comes from a number of studies which suggest that some of the inhibitors of protein synthesis discussed above also interact with the cholinergic system. Puromycin is a non-competitive inhibitor of AChE and competes with post-synaptic nictinic receptors; cycloheximide and anisomycin also inhibit AChE (Moss and Fahrany 1976; Zech and Domagk 1976).

Nonetheless if Deutsch is essentially correct in assuming that alterations in post-synaptic sensitivity are responsible in some way for LTM then more recent findings about experiential modulations of muscarinic receptor are quite exciting.

On a somewhat more theoretical plane, Stent (1973) argues that when a post-synaptic neurone fires, receptors are lost from the membrane in regions distant from the received input. Hence active synapses retain their receptors but inactive ones lose theirs. Extending this idea Davis (1976) has postulated that a neurone mediating an UR in a classical conditioning paradigm will receive active inputs from those neurones involved in the US and inactive inputs from the neuronal arrays responsible for all possible CS's. These non-functional synapses are depleted of receptor sites as a consequence of post-synaptic membrane potential reversal resulting from depolarization. Receptor associated with active synapses are not destroyed because the degree of membrane reversal is 'clamped' by the action of neurotransmitter. The notion of receptor destruction is based on the abolition of heightened sensitivity to ACh in denervated muscle that is brought about by direct electrical stimulation (Lømo and Rosenthal 1972). The problem that the non-functional synapses have no receptor sites to be 'protected' by a change in input is easily overcome by speculating not receptor destruction as a result of depolarization overshoot, as Davis does, but a readily reversible process such as displacement or conformational change of receptors. The contention that this model cannot account for extinction is also over ruled by this consideration; in any case it seems likely that extinction is mediated through alternative neuronal arrays. What makes this sort of model attractive is that it predicts that the occurrence of the US between US-CS pairings should interfere with acquisition. In other words it supports a correlatitive model of reinforcement (Rescorla 1967, see 1.3). The key difficulty with this hypothesis currently is that there is no convincing evidence showing just how an overshoot of the action potential leads to altered receptors (for a discussion of this point see Strumwasser 1976).

An alternative way of modifying synaptic efficacy might be to alter the nature of proteins which are not directly part of the neurotransmitter system, but which may be part of the structural matrix of the presynaptic membrane, the junctional lattice or post-synaptic density. It might be, for example, that conformational changes in a receptor protein are only made possible by adjacent cytoarchitectural remodelling.

The Goteborg group clearly see a role for S100 protein (Haljamae and Lange 1971). Their contention is that increased amounts of intracellular Ca^{2+} which seem to accompany training, alters the conformational state of S100 protein at the synapse and so, perhaps by increasing the area of contact of pre- and post-synaptic membrane, modulates synaptic efficiency.

It appears that while some of the evidence is conflicting, and most is, at best, only tentative and suggestive, in general the neurochemical viewpoint outlined supports, and in part extends the ideas of learning which have been formulated by other neurobiological disciplines. This review has deliberately ignored certain aspects, for example the possible role of neuropeptides in learning and related processes such as motivation since studies in this field, fascinating though they are, are still at too early a stage to make model building sensible. As it is, neurochemical models remain deficient in several fundamental respects. It has been possible to define modulations in a wide variety of cellular processes which accompany learning like hyperpolarization, modification in cation pump functioning with attendant amino acid transport, transcription, translation, post-translational regulation and alterations at the synapse, but there is only ignorance as to how these events are linked. Some clues may have been provided by the role of noradrenergic arousal mechanisms in initiating consolidation. Whilst many of the transient changes seen in ion movements, protein synthesis and the like seem to reflect acquisition and consolidation, and the longer lasting biochemical changes at the synapse, together with altered morphologies of particular neurones may represent storage, there is no knowledge about how such modifications of connectivity translate into selective, and, in the human case at least, directed, recall.

CHAPTER 2

IMPRINTING BEHAVIOUR AS A LEARNING MODEL

2.1 Imprinting as learning

Precocial birds are those that are capable of leading an independent existence within a few hours of hatching. Such species will, without parental nurturing, feed, move and respond appropriately to a variety of normal stimuli, which implies that hatching corresponds to a period late in the ontogeny of the bird. Precocial birds, like the domestic chick, exhibit a behaviour known as imprinting in which the neonatal chick becomes socially attached to any visually conspicuous moving object and will learn to recognise it and so to distinguish it from any novel stimuli. In the wild the first such object to which the young bird is likely to be exposed is the hen and during the development of this social preference the chick approaches and follows her and as a result gains proximity to warmth, food and is afforded a measure of protection from predators; all of which clearly increase the likelihood of the survival of the chick. Lorenz (1935, 1937) argued that imprinting was an important determinant of later mate selection, and since birds do not mate with their parents, it was concluded that imprinting conferred a knowledge of species but not necessarily the ability to discriminate individuals. However it is now believed that sexual imprinting is distinct from and occurs later than filial preferences (Schutz 1965). Clearly in dimorphic species, such as the mallard duck, for the sexual preference of the female to be influenced by filial attachment would be maladaptive, and in the semiprecocial gulls, adult birds will sometimes eat strange chicks in the colony; it would therefore be of survival value for chicks to recognise individual parents.

From the view point of animal behaviour or comparative biochemistry, chick imprinting may be worthy of study in its own right but a prime concern of this thesis is the applicability of this particular kind of behaviour to elucidate something of the nature of the biochemical processes which accompany, and hence may underlie learning generally; that is in both animals and the human. This is a difficult task, as it is in just the field of behavioural neurochemistry where one might expect to find the greatest differences between species, given both the inherent complexity of the nervous system, and that it is in large measure through behaviour that animals successfully adapt to the transient changes of circumstance in which they find themselves.

This chapter, then, is primarily addressed to arguments concerning the suitability of visual imprinting in the chick as a model of learning in general, for if this is the case an understanding of the biochemistry of the process takes on a far wider significance. In order to demonstrate this altogether more global role for imprinting it is mandatory to answer two fundamental issues. Firstly is imprinting a unique characteristic of precocial birds or can it be seen as a phenomenon not essentially different to other kinds of learning? Secondly what characteristics of imprinting is it necessary to understand and optimise in order to translate a naturally occurring behaviour into the artificial situation of the laboratory? This question, while somewhat more pragmatic than the first is equally important, since the answers must guide the design of imprinting experiments. These two questions will be discussed in turn.

It has been argued that imprinting is a unique process and distinct from learning (Lorenz 1935, 1937; Hess 1959 and Eibl-Eibsfeldt 1975). Reasons advanced for its uniqueness have been its rapidity, durability, irreversibility and dependence on a critical period.

Other types of learning can be shown to be far more rapid; for instance, one trial passive avoidance learning in newly hatched chicks, or durable, for example, it has been shown that pigeons will correctly perform an operant conditioning task on which they had last been trained 4 years previously. Neither is it tenable to regard imprinting as a special case of learning on the basis of its irreversibility or the existence of a critical period; the only peculiar feature it might be said to display is the context in which it occurs (Bateson 1966). It is worthwhile to consider in more detail some of the experimental evidence which has helped shape modern notions about the nature of imprinting.

2.1.1 Durability

Earlier notions of imprinting regarded it as irreversible (Lorenz 1935, 1937), a view that seemed to be supported by evidence that choice of mate could be influenced by early learning. At least one study (Salzen and Meyer 1967) has disproved the idea of irreversibility. Chicks were exposed to coloured balls for 3 days, after which they were tested by a two choice discrimination test to ascertain that imprinting had occurred. For the next 3 days the birds were trained with balls of a different colour, and on retesting showed a complete reversal of preference. This,

of course, militates against the primacy-recency hypothesis of Hess (1959) which held that the initial imprinting experience over-rides all subsequent experience in influencing behaviour. Interestingly the period of reversal training was long outside the originally accepted critical period for imprinting. Furthermore it took place as rapidly as the initial training, which suggested that there was no greater fear of the novel object to overcome before reversal training could occur.

2.1.2 Sensitive period

The term 'critical period' was applied by Lorenz (1935) to the immediate post-hatch interval which was thought to be the only period when birds would reliably imprint. It implies a period fixed by ontogeny. One typical experiment trained mallards of various ages on a moving surrogate and then tested them between 5 and 70 hours after training, and so determined a critical period that lasted from 5-24 hours with a definite peaking at 13-16 hours (Ramsay and Hess 1954). A well defined peak emerged in an experiment which synchronised duck eggs to the same developmental age, between 648-659 hours post-incubation (Gottlieb 1961), and similar results have been obtained in a chick study which investigated flicker frequency preferences in birds trained on a flashing light (Simner 1972). Gottlieb thus regarded developmental age as the important determinant of the onset of the critical period, which suggested that maturational rather than experiential changes are involved. A more recent experiment (Landsberg 1976) kept the time from training to testing constant so that the age of testing varied with post-hatch age, and with developmental age, and found that both had significant effects on the onset of the sensitive period.

Considerable evidence exists to counter this view of an immutable critical period. By manipulating environmental and training variables the period during which imprinting can occur can be considerably extended. Partly for this reason the term sensitive period is now extant in the literature, and during this period there will be , 'a gradual change in the probability that imprinting will occur depending on the conditions of rearing and test procedure' (Kaufman and Hinde 1961). Domestic chicks, socially reared, when tested for approach and following responses for the last 5 minutes of a 30 minute training period, seemed to be most responsive in the first 6 hours post-hatch, less responsive at 6-54 hours, and no birds responded after 54 hours. Interestingly, although the proportion of birds that were responsive decreased with age, for those which did respond there was an increased strength of following with age which may have resulted from an improved ability to recognise

the characteristics of the stimulus, or possibly better locomotor skills. Using intermittent light as an imprinting stimulus one study showed that approach tendency was found to be stronger in chicks 24 hours old than one week old; however one week old birds did respond (James 1960a). Other studies have confirmed this; chicks will follow a moving object when 5-7 days old on first presentation of the stimulus (Salzen and Sluckin 1959) and ducklings reared apart from their hatch-mates will follow after 10 days (Fabricus and Boyd 1954). At 7 days post-hatch isolated chicks would approach an intermittent light whereas socially reared birds would not (James 1960a, 1960b). Results consistent with this have been obtained with a moving object and the conclusion drawn was that imprinting could itself terminate the period of general responsiveness - a view which implies irreversibility (Sluckin and Salzen 1961). It seems likely that socially reared birds imprint on each other and with their preferences thus restricted they will respond less readily to another stimulus (Guiton 1959).

It has been suggested that maturational changes are important in the termination of the sensitive period. A developmental increase in fear has been postulated as a result of the observation that active avoidance learning is not exhibited by chicks in their first day of life, but is acquired later (James and Binks 1963). However in Peking ducks it is very difficult to elicit fear responses even at the close of the sensitive period (Guiton 1961).

Perhaps if 'endogenous' fear is ruled out, fear may increase as a result of the birds' early experience. Fear is clearly present in domestic chicks after 3 days post-hatch (Spalding 1873). The major stimulus to generate avoidance responses in older birds has been shown to be strangeness (Schaller and Emlen 1962) and it is known that novel stimuli evoke fear (Moltz and Stettner 1961a). The contrast between the familiar and the novel is increased by greater times of training with the imprinting stimulus; in other words, as imprinting proceeds, the chick restricts its preference to a familiar object, presumably because it learns to discriminate between this and any novel object, the latter of which generates fearfulness. Moreover, exposure to a novel environment will enhance following of a familiar object (Sluckin 1960; Sluckin and Salzen 1961). Socially reared chicks showed more avoidance than isolated birds when exposed to a moving object on the second post-hatch day (Salzen 1962) maybe because when isolated the socially reared bird is effectively in a novel, fear producing environment.

Clearly while species differences exist in timing, and while a particular developmental state may make imprinting occur more readily, the experience of young birds plays a key role in determining exactly when imprinting takes place.

2.1.3 Strength of attachment

Hess (1964) thought that the strength of imprinting was related to the distance travelled by the bird in its following responses, and so the amount of effort expended by the animal. Keeping a constant exposure time, ducklings were tested by a two choice discrimination test to determine the strength of imprinting of birds made to run different distances (Hess 1959). Birds which ran the greater distance seemed to be more strongly imprinted. However the birds were made to run the greater distances by increasing the speed of the stimulus, and it is conceivable that the more rapidly moving a stimulus is the more effective an imprinter it becomes (Sluckin and Salzen 1961). Possibly the ducklings had less visual experience of the slower moving stimulus because they spent a larger proportion of time nestling up against it (Bateson 1966). Birds are known to imprint less well to a stimulus 7 inches distant than one 14 inches distant (Moltz 1960). Meprobamate in chicks acts as a muscle relaxant, so reducing proprioceptive input, and while it does not impair the learning of a colour discrimination task for a food reward, it will impair the acquisition though not the retention of imprinting (Hess 1957). This drug also produces drowsiness (Hess 1964), though it is not immediately apparent why this should have a differential effect on the two learning tasks. Based on these and other studies Hess attempted to formulate a mathematical model of what became known as the 'Law of Effort!'

Many studies have found imprinting to occur when the birds have been prevented from following (Moltz, Rosenblum and Stettner 1960; Smith 1962). In a study involving 106 Ross 1 domestic chicks (Bateson and Jaeckel 1974) measurements of the birds' activity were made throughout a total of 72 minutes training and were found to correlate weakly with a preference for the familiar, but the authors concluded that the result was too tenuous to be the basis for a law.

The strength of attachment appears to be a function of the time of exposure to the stimulus (Salzen and Sluckin 1959; Bateson 1974a). Recent work has shown that birds can exhibit social attachment to more than one object (Jaynes 1957; Guiton 1959), although the timing of presentation of the stimuli is important. If two dis-

similar stimuli are presented in rapid alternation, chicks behave as if they regarded both stimuli as belonging to a single perceptual classification, but if the stimuli are presented alternately, in half hour exposure periods, the birds behave as if the stimuli were independent (Chantry 1976). It has been proposed that the chicks' recognition of the hen as a single individual comes about because although she displays many dissimilar aspects of herself, she does so in rapid succession (Chantry 1974).

2.1.4 Learning theories

In addition to the claims for uniqueness advanced by Lorenz, Hess was concerned to demonstrate that imprinting could not be regarded as a type of association learning (Hess 1959, 1964). He thought that spacing of training sessions, rather than massed training was more effective in association learning, but believed the converse to be true of imprinting. However, at least one study (Fischer 1966) has found distributed rather than continuous practice to be more effective in imprinting. In association learning whatever has been the most recently learned has the greatest influence on behaviour, however with imprinting Hess claimed that primacy (the first experience) over-rides recency. This is in effect another way of saying that imprinting is irreversible, and follows logically from the notion of a sharply defined and inflexible critical period. Moreover, while with association learning painful or aversive stimuli reduces the likelihood of learning occurring, with imprinting Hess thought that the reverse was true.

Perceptual learning

Many other authors have accepted that imprinting is learning but have regarded it as non-reinforced (Sluckin 1962, 1972; Salzen 1970; Sluckin and Salzen 1961; Bateson 1966, 1971). A perceptual or exposure learning model suggests that the bird learns the features of a stimulus merely as a result of exposure to it, and so builds a 'neuronal model' of the stimulus. Mismatch between this percept and the ongoing sensory input is supposed to be responsible for aversive responses; with fear coming about as a result of novelty recognition (see 2.1.2).

At least one experiment has set out to test the perceptual learning hypothesis. Chicks were reared and trained in either a large or a small cage and subsequently tested in a large chamber. This meant that for the small cage trained birds the testing situation was relatively more novel than for those trained in the large cages

(Brown and Hamilton 1977). Even in one experiment which maintained the perceptual disparity in size, but used transparent partitions to make the actual cage sizes equal, so as to control for differences in the opportunity for development of locomotor skills, there was a significant difference between the groups with regard to the time taken to approach the imprinting stimulus. This provides support for a perceptual learning hypothesis. What these authors observed however ran counter to the usual notion that environmental novelty, in generating fear, increases the following of the familiar object. The birds reared in the small cages, for which the test situation was most novel, had longer latencies than those trained in the larger cages. All groups of birds were immobile on first exposure to the test chamber, indicating marked fear, and it was postulated that the relationship between fear and approach responses can be represented by an inverted U shaped curve; that is, sufficiently high levels of fear will inhibit approach. It is worth noting that approach latencies decreased with the number of trials, which is reminiscent of conditioning.

The perceptual learning hypothesis has been criticised (Hoffman and Ratner 1973). In an experiment with isolated chicks, distress vocalization was at a minimum when birds were in a familiar environment but high when exposed to a novel one. This was taken as indicating that the birds had learned the characteristics of their environment. This has been documented previously (Bateson 1964); chicks raised in patterned pens approach a similarly patterned stimulus more than a dissimilar stimulus (which incidentally is suggestive of exposure learning). In the Hoffman and Ratner experiments removal of the imprinting stimulus elicited distress calling in both environments, presentation of the imprinting stimulus in either reduced such vocalization. That distress calling occurred in the familiar environment after removal of the stimulus indicated that a social bond had been formed to the moving object, and presumably that a much weaker, if any, attachment had been made to the familiar environment. However exposure to the novel environment also elicited distress calling either because novelty generated fear (although the birds were only 17 hours old - an age when fear is not usually displayed) or because the animals had been removed from the familiar environment which it had not only learned to recognise, but had also become filially attached to. If this latter is true then the assertion that only the development of 'neuronal models' to certain classes of stimulation, such as that provided by moving objects (see Sluckin 1962) reflects the formation of a social bond, cannot be true.

If regarding imprinting as a non-reinforced type of learning seems to give rise to paradoxes, can an association learning approach by any more successful?

Conditioning

The view that imprinting is non-reinforced learning has been widely disputed. James (1959) regarded imprinting as classical conditioning. Moltz (1960) thought that the low anxiety state experienced by the newly hatched bird becomes associated with a moving object, which thus becomes a reinforcer for a conditioned response (CR) of low anxiety and instrumental in generating approach and following. This keeps the bird in the close proximity of the stimulus where it continues to learn the characteristics of the stimulus. Rajecki (1973) regarded approach and following as an unconditioned response which can be conditioned. Does the imprinting stimulus act as a reinforcement in this conditioning? What is required of a reinforcer is that its presence contiguously with the conditioned stimulus (CS) should increase the likelihood of the conditioned response occurring. Continued exposure to the imprinting stimulus does just that, since the longer the animal is exposed to the stimulus the more strongly will it subsequently approach and follow. It would appear then that the imprinting stimulus is both the UCS and the CS.

There is considerable evidence to support the view that the imprinting stimulus has reinforcing properties. Ten day old visually isolated ducklings can be shaped to peck a pole to get a 10 second presentation of an imprinting stimulus (Hoffman et al 1966). Withdrawal of the stimulus whenever a duckling attempted to follow caused a decrease in this behaviour (Hoffman, Stratton and Newby 1969). An environmental stimulus which is not part of the imprinting stimulus but consistently paired with it can become a CS capable of reducing distress vocalization (Hoffman et al 1972). Intermittent light coupled with a suspended polythene ball (James 1959) during ten 5 minute training sessions caused chicks to approach and follow the ball, subsequently presented alone, significantly more than birds presented only with the ball during training. Moreover, an imprinting stimulus has been found to be reinforcing even during a birds' first exposure to it (Bateson and Reese 1968; Hoffman, Eiserer and Singer 1972) which suggests that development of the fear of novelty plays no part in this process.

If imprinting were conditioning, it should be possible to extinguish it. An attenuation of approach and following responses towards an object previously paired with

a flickering light have been found (Abercrombie and James 1961), however this does not necessarily represent extinction. Clearly the birds' response is altered, but approach and following are not an essential for imprinting (although such a response may be the only way of knowing that imprinting has occurred). Perhaps less ambiguous was a study using 5-10 day old ducklings in which an imprinting stimulus was found to be reinforcing to birds initially imprinted on an alternative stimulus (Gaioni et al 1978), most of the birds switched preference to the second stimulus and it could be argued that this represents, at least in part, an extinction of the initial training.

A conditioning model predicts that punishment, that is, negative reinforcement, given contiguously with the stimulus should reduce approach and following. Filial behaviour is actually increased by periodic shocks in the presence of the stimulus at 18 hours after training (Kovach and Hess 1963) possibly because the shocks increase the novelty of the environment. However at 32 and 48 hours after training following was hindered by punishment. Another study (Barrett et al 1971) found that if the shock was made contingent on some operant of the bird i.e. following, then filial behaviour reduced.

Stimulus generalization, predicted by a conditioning model, but more difficult to reconcile with a perceptual learning model, is known to occur since previously imprinted subjects, when enforcedly exposed to a novel stimulus imprint more rapidly than unimprinted controls. Experiments in which chicks were exposed to one of three stimuli which differed only slightly from each other indicated that birds will generalize their preferences although they will retain the greater preference for the stimulus of which they have previous experience (Jackson 1974). This generalization probably comes about because in the earlier stages of imprinting birds will work for slight novelty (Jackson and Bateson 1974). Chicks between 14-22 hours post-hatch, were trained, after a 50 minute exposure to a diffuse light source, with either a red or yellow checkerboard patterned flashing light for 15, 30, or 60 minutes. Three minutes after training had ended the birds were placed individually into an operant conditioning box where they could press one of two pedals in order to obtain a view of the novel stimulus. Pressing the alternative pedal had no effect. While chicks after 60 minutes training pressed both pedals equally i.e. they showed no inclination to work for novelty, both the 30 minute and to a greater extent the 15 minute groups pressed the pedal giving access to novelty significantly

greater than chance.

The suggested biological role for this is that chicks earlier on during imprinting are more likely to work to see different views of the hen, and so come to recognise her characteristics from all possible angles and postures (Bateson and Jaeckel 1976).

The imprinting stimulus then, is clearly reinforcing. Rajecki (1973) makes the point that the reinforcing properties are given mainly by the movement (the UCS) and filial behaviour generated by this becomes associated with the more static aspects of the stimulus such as shape and colour, which thus take on the role of CS by a classical conditioning process. The suggested underlying physiology to this is that the motion fires a population of movement sensitive cells which elicits filial behaviour whereas other aspects of the stimulus, initially neutral, i.e. shape and colour, fire different cell populations. Reinforcing properties have been found for all flicker rates between 1.5-11.5 flashes per second, though the midpoint of the range has been found to be most effective (Simner 1975). It is interesting that flicker frequency preference seems to be innate, since it cannot be modified by earlier post-hatch experience of different flicker rates (Simner 1974). A possible problem with Rajecki's interpretation is that birds will imprint eventually to stationary stimuli (Gray 1960).

It would seem that evidence has been adduced in support of both perceptual learning and conditioning theories of imprinting. The current state of experimental knowledge does not permit an unambiguous distinction, possibly because imprinting involves aspects of both, or maybe because the classification of learning in this manner is artifactual and says nothing about what might be common underlying processes. Despite the problems about the conceptual framework into which imprinting might be fitted there can be little doubt that it is neither a unique behaviour or a special case of learning. It has far greater similarities than distinctions with other sorts of learning and therefore should be of general applicability to the neurochemistry of the learning process.

2.2 Stimulus and environmental characteristics

Newly hatched chicks apparently show no innate preference for the visual properties of the hen (Reese et al 1972) although at face value one study (Case and Graves 1978) suggests otherwise. Exposure to a conventional imprinting stimulus for 20 minutes did not prevent chicks one day later from showing a marked pre-

ference for a surrogate hen during a two minute, two choice, discrimination test. It appears that the surrogate hen was by far the better imprinter, although since birds trained on the surrogate were tested simultaneously, whereas those trained on the imprinting stimulus were tested 24 hours after training, direct comparison may not be valid. But the most likely reason for the finding was that the chicks exposed to the imprinting stimulus, having barely imprinted, responded to the surrogate as if they were naive birds.

Whatever the case it is clearly important to establish exactly what features are important as releasers of imprinting behaviour, and under what conditions, so as to expedite an optimal experimental design.

2.2.1 Stimulus colour

Colour preferences have been studies by observing the approach and following responses generated by moving coloured spheres (Schaefer and Hess 1959). On the first day post-hatch chicks responded, from best to worst:- blue, red, green, orange, grey, black, yellow, then white. However the situation is more dynamic than this suggests since an experiment which tested birds each day from one to five days post-hatch found that a good response to red peaked on day 2 and then tapered off, (Gray 1961). In an investigation which compared the effect of exposure of birds to either a pen with coloured walls, imprinting on a coloured, moving, perspex square, or a colour recognition dependent conditioning procedure, naive birds showed an initial preference for red or yellow over blue in a two choice discrimination test (Taylor, Sluckin and Hewitt 1969). The three training procedures were equally effective in producing a shift of preference towards the familiar colour, except in the case of blue. In other words, training on the initially non-preferred colour was less effective than training on the initially preferred colour. The equal effectiveness of the three types of training and the fact that conditioning was not essentially different in determining the subsequent behaviour of the animal than the imprinting again serves to highlight the difficulties inherent in attempting to classify learning.

2.2.2 Stimulus movement

Visual flicker, which is thought to simulate movement on the avian retina, has been shown to be effective in releasing approach and following in domestic chicks (James 1959; Smith 1960; Smith and Hoyes 1961). Although imprinting on stationary objects

has been reported, a far greater number of trials were required to meet the criteria for a stationary than for a moving stimulus (Gray 1960; Abercrombie and James 1961). A study comparing the effect of a stationary object with the same object when moving, concluded that movement itself can act as an unconditioned stimulus in suppressing distress vocalization (Hoffman, Eiserer and Singer 1972). Clearly movement or visual flicker whilst not absolutely essential to imprinting certainly enhances its acquisition.

The frequency of flicker seems to be important. A preference for 4 flashes per second has been demonstrated in chicks between 512 and 530 hours after the onset of incubation (Simner 1972) and the important parameter has proved to be the rate of flicker as opposed to the interval between successive flashes (Simner 1976).

2.2.3 Stimulus intensity

Following on an idea by Schneirla (1959) that early on in development stimulus intensity is more important than its specific characteristics, Moltz (1963) argued that low or decreasing levels of stimulation would produce following, but intense levels produce avoidance responses. This has subsequently been refuted (Salzen 1970).

Alternatively it might be regarded that birds be differentially sensitive to stimulus intensity according to their age; younger birds requiring higher intensity to elicit the same degree of behavioural response. Certainly the visual system of ducklings continues to develop after hatching and it has been found that chicks become more easily aroused as they get older, at least for the first few hours. However using the initial approach tendency of chicks aged between 12 hours and 7 days to a flickering light of variable intensity, it was found that birds of all ages showed a preference for the same range of luminosities (Kovach 1970).

2.2.4 Social rearing

Chicks reared socially for three days post-hatch do not approach and follow a moving object (Guiton 1958, 1959) possibly because the birds imprint on their hatch-mates. Certainly social rearing has been shown to attenuate the chicks' response towards intermittent light (James 1960b; Reese et al 1972).

2.2.5 Environmental temperature

Ambient temperature seems to be an important determinant of imprinting behaviour. A high rate of distress calling is elicited by low temperature (Kaufman and Hinde 1961), and the following response is delayed (Salzen and Tomlin 1963). Even more fascinating is the finding that cold stress alters the usual preference for blue over green (Davis and Fischer 1978).

2.2.6 Atmospheric pressure

Even environmental factors during incubation may be important in determining the future behaviour of chicks. For Ross chicks the barometric pressure on day 12 of incubation correlates negatively with the approach activity on the first post-hatch day and this could certainly account for some of the great variability observed between different hatches of birds (Bateson 1974b). It has been suggested that the physiological mechanism operating here is that the altered rates of gas transport across the egg shell affect the partial pressures of gases in the blood, to which the developing nervous system must be exquisitely sensitive at this time.

2.2.7 Light exposure

Chicks from eggs incubated in the light show a less marked tendency to approach and greater avoidance behaviour than those from eggs incubated in darkness (Dimond 1968). Prior experience of diffuse light has been shown to improve subsequent imprinting (Bateson and Seaburne-May 1973), one trial passive avoidance learning in day old chicks (Cherfas 1977), and colour discrimination for a heat reward (Cherfas 1978). It appears that prior post-hatch visual experience 'switches on' processes which makes visual learning tasks easier to acquire, so if the bird is taken straight from the dark to the learning situation, the first few minutes are taken up not by learning but by this visual 'priming'.

2.2.8 Circadian rhythmicity

Chicks kept in isolation for 1 to 4 days post-hatch when tested spent more time in the vicinity of a hen at midnight than at other times of the day (Gray 1962). Since ground nesting birds are more vulnerable to attack by predators at night, it is possible that this rhythmicity might have conferred a selective advantage during evolution.

That this rhythmicity in the chicks' behaviour might be brought about by activity

changes in the hen (Gray, Sallee and Yates 1964) is unlikely, since reduced activity (chickens are diurnal rather than nocturnal animals) would be expected to curtail approach and following responses in the chick.

2.2.9 Auditory imprinting

A neonatal chick, while responsive to a wide range of sounds, has a marked innate preference for the parental call appropriate to its species (Gottlieb 1965) and this preference could not be altered by prior exposure to other calls. However when naive domestic chicks were given a choice between two non-parental calls they showed a distinct preference for that call which they had experienced earlier indicating that some measure of auditory imprinting had occurred. One possibility considered by Gottlieb (1966) to explain the innate preference was that chicks would imprint on their own calls, but the neonatal call proved to be not very effective in eliciting following responses when compared with the maternal call.

A second possibility might be that chicks imprint to the maternal call while in ovo. Domestic chicken eggs exposed for the last 6 days of incubation to patterned sound produced neonates which followed a moving model longer when it emitted the same sound than when it emitted a novel sound or remained silent (Grier, Counter and Shearer 1967). If prenatal auditory imprinting does occur then it must represent the earliest stage in the development of the maternal - neonatal attachment and might be thought of as 'setting the stage' for and enhancing the efficacy of subsequent visual imprinting.

2.3 The context of imprinting

It appears that there is no prima facie case for regarding imprinting as in principal different to any other kind of learning. One might agree with Bateson (1966) that the only unique feature about imprinting is the context in which it occurs, although this may be because it is only in precocial birds that this kind of behaviour can be cleanly dissected from confounding factors.

The possibility emerges for consideration of a wider context in which 'imprinting-like' learning may occur. In precocial mammals, such as the guinea pig, following responses have been elicited by exposure to a rotating white octagonal block (Shipley 1963) and since then other workers have collected evidence which gives support to the notion (Sluckin 1968; Sluckin and Fullerton 1969; Harper 1970). More problematic

is the question of imprinting in altricial animals; since by definition approach and following responses are impossible at an early age in such species. Nonetheless studies using dog pups have attempted to show that later socialization of dogs with humans would occur independently of food reward and therefore was related simply to exposure rather than conditioning (Brodbeck 1954; Scott 1962). Sluckin (1972) regards the attachment of infant monkeys to their mother or mother surrogates as an imprinting-like process, but here certain surface textures with which the neonate finds itself in contact seem innately attractive, much in the way that visual flicker is to the chick. If this is a valid interpretation there is no a priori reason to reject imprinting as being a component, of early learning in the human infant, although the evidence for such is anecdotal and extremely speculative and will not be discussed here. What emerges, if it is accepted that human imprinting does occur, is that its role must be somewhat different to that in the chick. Clearly imprinting in humans cannot be seen as a means of increasing the survival chances of the neonate by maintaining close proximity between it and the mother, but the formation of a social bond, mediated by early learning which need not be reliant on externally reinforcing contingencies, may be important to survival later in life when the infant is mobile and, given the findings concerning the effects of maternal deprivation in primates, might also contribute to the stability of the family unit from one generation to the next.

One might speculate that imprinting involves a unique 'set' of neurones, by virtue of the particular characteristics of the stimulus and the developmental state of the animal, but that the changes occurring at the cellular level are common to all types of learning. In other words, the 'circuitry' determines the type of learning, and so the appropriateness of the response which an animal makes in a given situation, but the plastic changes which establish the pattern of that circuitry are brought about by identical biochemical modulations.

CHAPTER 3

NEUROCHEMICAL CORRELATES OF IMPRINTING

3.1 Introduction

Imprinting is a valid and potentially useful model of learning as it occurs very early in life, before many other significant events have had the opportunity to exert their neurochemical 'stamp'. The visual experience of the chicks can be restricted to all but the imprinting stimuli, and for the short time that this is necessary it is unlikely that the animals will suffer sequelae contingent upon visual deprivation. Birds isolated in small pens have little opportunity to experience a variety of tactiles cues, or to develop a wide range of locomotor skills and, moreover, food and water can be withheld since the yolk sac can sustain the neonatal bird until it is 48-60 hours old. Such chicks then are naive.

Many of the experiments done to investigate the neurochemical correlates of imprinting have produced results which parallel and thus add weight to the work on other species which have used different paradigms and methodologies. However it has often been possible to manipulate imprinting behaviour in such a way as to be more than usually certain that the observed effects are learning related and in addition some of the work has examined biochemical aspects not studied in any other learning situation.

3.2 Adenyl cyclase and cAMP

Because I have argued that the changes seen in the adenyl cyclase cAMP system which occur in the first hour of exposure to an imprinting stimulus are more related to arousal than to learning, this data has already been discussed (1. 7).

3.3 RNA studies

The early investigations concerned with RNA metabolism in this laboratory used a circular arena in which chicks held in pens situated around the circumference were exposed to an orange flashing light 45 cm distant as an imprinting stimulus, or to a single light source in the form of a 60 W. bulb held 2 m overhead (Bateson, Horn and Rose 1972).

^{3}H-uracil incorporation was found to be enhanced throughout the brain in both imprinted and light exposed groups, compared with dark controls, after a 150 minute pulse occurring concurrently with their appropriate training conditions. A further experiment kept the same time from injection of precursor to killing but trained birds either for 38 or 76 minutes in a symmetrical design within that time. This revealed a far more specific effect. Only in the dorsal forebrain at 76 minutes was there an increase in uracil incorporation in trained birds as compared with light and dark controls. Although total uracil pool changes also occurred in the dorsal forebrain (roof) region it is difficult to assess their significance because later work showed that considerable exchange of tritium occurs from precursor to water during long in vivo pulse times (Haywood, unpublished work). Two processes then were defined. One was a late, generalized synthesis of RNA in response to light stimulation, and the other an early, localized synthesis in response to exposure to the imprinting stimulus.

There are many possible explanations for this localized increase in uracil incorporation apart from learning, such as stress, arousal, attention, motor activity, or sensory stimulation. A series of three experiments were designed to eliminate, when considered together, these factors that are so intimately related to any learning situation.

The first such experiment took advantage of the peculiar anatomy of the avian visual system. The optic chiasma of birds comprises a complete decussation of the retinotectal pathway (Cuenod 1975) that is, the visual field of one eye projects exclusively to the contralateral side of the brain (Cowan, Adamson and Powell 1961; Karten et al 1973) making it possible to train one half of the brain while the other half remains naive. However the supra-optic commissure of birds is probably the homologue of the mammalian corpus callosum and mediates the interhemispheric transfer of newly acquired behaviours (Cuenod and Zeier 1967). Although transfer seems to depend on the nature of the learning, both one trial avoidance learning (Cherkin 1970) and imprinting in ducklings (Moltz and Stettner 1961b; Demarest 1977) appear to be rapidly transferred. To avoid this transfer of training between hemispheres the supra-optic commissure was cut creating a 'split-brain' chick. This neurosurgery was carried out on 12 birds which subsequently had one eye trained, when 19-28 hours old, with a total of 60 minutes exposure to a yellow imprinting stimulus. Half of the birds had the left eye trained and half the

right, the contralateral eye being covered (Horn, Rose and Bateson 1973). The pulse time was 150 minutes as before.

Birds were tested with each eye separately by a two choice discrimination, which gave the animals a choice between the familiar and a novel stimulus. (The rationale of this test is discussed in 4.2). It indicated that the trained, but not the untrained, half of the brain had learned a preference for the familiar. In the forebrain roof when the right side of the brain was trained, then the trained side showed a 15% greater incorporation of uracil into RNA than the untrained side. Essentially this experiment uses an animal as its own control and suggests that the biochemical change could not be accounted for in terms of differential levels of circulating hormones, for example as a result of stress, or in terms of changes in motor activity.

The situation is complicated by the fact that when the left side of the brain is trained there were no differences between sides. Tentatively this dissimilarity between left and right sided training was explained as being the result of asymmetry in the surgical procedure. It is difficult to see what processes might be involved; uracil pools were unaffected by training either side.

One possibility might have been that the different biochemical response of the two sides represented lateralization of memory fixation, as has been described in the chick by Rogers et al (1980). This is ruled out however as the authors state that the left hemisphere trained birds showed a clear preference for the familiar stimulus. It is relevant here that no lateralization was found for the one trial passive avoidance task (Bell and Gibbs 1977).

Equally difficult to explain is the finding that monocularly trained, intact chicks failed to show a difference in uracil incorporation between trained and untrained sides. One explanation given by the authors, namely that there was insufficient light stimulation to generate the biochemical events cannot be true, because monocular training of the ' split-brain' chicks (which had only 60 minutes training as compared with the two intact groups which received 76 and 155 minutes) did produce an asymmetry. An alternative view, since it is known that chicks show interhemispheric transfer of imprinted behaviour (Bateson, unpublished data) might be that the biochemical sequelae of training are transferred to the contralateral hemisphere, so negating the differences. However with one trial learning, which

is also transferred (Cherkin 1970), the transfer does not involve the encoding of memory in the untrained hemisphere; injection of CXM or ouabain into the forebrain opposite the trained eye two minutes after training produced amnesia one hour later, but injection into the ipsilateral forebrain did not, showing that the engram exists only in the trained hemisphere (Bell and Gibbs 1977). Two possibilities thus emerge; either imprinting and one trial learning are dissimilar in that monocular training results in bilateral storage in one but not the other, which assumes that RNA synthesis does underlie consolidation, or alternatively that for both sorts of learning monocular training results in unilateral storage. and it is difficult to see what the asymmetrical uracil incorporation changes in the split brain preparation represents.

Clearly the two halves of the brain differ in the amount of visual stimulation which they receive and it is known that visual attention causes localized changes in cerebral blood flow (Bondy, Lehman and Purdy 1974) which could have caused an asymmetry in precursor availability.

In order to obviate the differences in sensory stimulation experienced by birds at a time when they are incorporating the radioisotope, a second experiment trained birds for different times on the first day, from 20-240 minutes but gave all birds 60 minutes training on the second day, when a ^{3}H-uracil pulse was given. The assumption made was that the amount that the chicks learned on the second day would depend on their previous experience of the stimulus. Since the degree to which birds exhibit imprinting behaviour correlates with the time of exposure to the stimulus it was argued that the extent of further learning on day 2 would diminish as the length of training on day 1 was increased. If uracil incorporation is related to new learning the hypothesis predicts that it should increase inversely with the length of training on day 1. Again using a two choice discrimination test it was shown that preference for the familiar did increase with length of training on day 1, and that as length of exposure on the previous day increased, then the incorporation of uracil into the anterior dorsal forebrain decreased (Bateson, Rose and Horn 1973).

Chicks' behavioural responses to an imprinting stimulus are very variable, even between hatch-mates, and this property was exploited in the design of a third experiment. It consisted essentially of a correlation study which investigated how the

degree of ^{14}C-uracil incorporation related to preference for the familiar. 106 birds were trained for a total of 80 minutes on either a red or yellow flashing light, starting 15 minutes after the injection of precursor. At 21 minutes after the end of training birds were given a five minute test and then killed. The total pulse time was 120 minutes. The incorporation of uracil was found to be positively correlated with the preference for the familiar only in the anterior dorsal forebrain (Bateson, Horn and Rose 1975).

Additionally with shorter approach latencies, which in this experiment correlated well with a measure of activity (Bateson and Jaeckel 1974), incorporation into anterior dorsal forebrain, ventral forebrain (base), and midbrain was reduced. This was believed to reflect a 'sink' effect, in which precursor was sequestered to skeletal muscle, presumably as a result of cholinergic dependent dilation of muscle arterioles in the more active birds.

The indications are, then, that the synthesis of RNA, if this is what increased uracil incorporation represents, is necessary to learning, rather than being generated by some less specific concomitant.

Strong supporting evidence for an increase in RNA synthesis was the finding of increased activity of RNA polymerase in a nuclear fraction of the forebrain roof of imprinted birds, compared with light exposed and dark controls. This was a brief phenomenon, marked after 30 minutes of exposure to the stimulus; but it was not present at 15 minutes and had disappeared by 45 minutes. Because of the rapidity of the effect it was regarded as being a stimulation of activity of pre-existing enzyme rather than de novo synthesis. The in vitro activities certainly indicate the maximum possible activity at the chicks' death but may not reflect the actual degree of stimulation in vivo, since the various species of this enzyme are differentially ion dependent. The time course and localization of this event accords well with the other changes seen in RNA metabolism (Haywood, Rose and Bateson 1975).

It is relevant here that the inhibition of the specific RNA polymerase II, by the alkaloid α-amanitin, has been found to produce amnesia for conditioned avoidance tasks in both rats and mice (Montanaro et al 1971; That and Lindell 1974).

Clearly the dorsal forebrain seems to be the region that responds to some aspect of the imprinting situation. An autoradiographic study was undertaken to define better the anatomical location (Horn, McCabe and Bateson 1979). A two day experiment with differential training on day 1 (either 45 or 80 minutes) was used so that all birds trained for 60 minutes on the second day would be initially either under or overtrained. On the second day 20 birds, matched in pairs, of one under, and one overtrained, according to activity during training on the previous day, were injected with ^{14}C-uracil, trained, and killed after 150 minutes. Autoradiography was carried out on serial coronal sections and indicated that differences in incorporation had occurred only in the medial hyperstriatum ventrale (MHV) of the dorsal forebrain (see Fig. 5).

3.4 Protein studies and precursor incorporation problems

Changes in the metabolism of proteins might logically be expected to follow altered RNA metabolism. For birds which hatched 6-9 hours before the midpoint of the hatch, 105 minutes exposure to the imprinting stimulus produced a substantial increase in the incorporation of ^{3}H-lysine in the forebrain roof, when compared with light or dark controls. This did not occur in any other brain region, neither did it occur in birds which hatched 6-9 hours after the hatch midpoint. It is important to note that experiments commenced at the same post-hatch age for both early and late hatchers, which indicates that this biochemical response was at least partly related to the developmental age of the chicks (Bateson, Horn and Rose 1972).

At this point it is worth bearing in mind that there are a number of serious interpretational problems with incorporation studies which have to do with the nature of the precursor chosen and the metabolic compartmentation of the precursor prior to its incorporation into the macromolecules. It is on the grounds of such problems that a number of incorporation studies have come to be heavily criticized (see Rose, Hambley and Haywood 1976).

Use of radioactively labelled precursor permits the detection of small changes in protein metabolism, because of the accuracy with which it is possible to measure radioactivity. Minute quantities of precursor can be used which are unlikely to disturb the system. Clearly the selection of precursor is important. For protein synthesis studies amino acids should be used, fragments of which are not appreciably incorporated into macromolecules other than proteins, such as nucleic acids

and lipids. Moreover amino acids can undergo metabolism to acetylCoA and so serve as energy donors in oxidative phosphorylation to a degree which is influenced by the nutritional status of the animal. Radioactivity in ^{14}C-amino acids would then find its way into Kreb's cycle intermediates. Recently in a study of protein metabolism during the learning of an operant conditioning task which examined the incorporation of leucine, lysine and methionine into protein (Hershkowitz et al 1975) training was shown to increase the catabolism of leucine to non-amino acid products and reduce the amount of label incorporated into brain proteins. This was found to be far more extreme for ^{3}H-methionine which lost 90% of its isotope into $^{3}H_2O$ in the first 20 minutes after the injection, while ^{35}S-methionine also lost ^{35}S into an unidentified component. Lysine was the most stable amino acid.

Clearly it is necessary to be cautious when comparing results from incorporation studies which have used different precursors. Also it seems reasonable to select for such studies an amino acid which undergoes the minimum of such metabolism so that the specific activity of the radiolabelled precursors changes little during the period of incorporation (pulse time), and that it is maintained at a tolerably high level to ensure sufficient labelling of the macromolecule during a short pulse time.

The fate of a parenterally administered amino acid is illustrated in Fig. 1, where it will be seen that a number of factors influence incorporation into protein. The availability of amino acids to brain cells is dependent on cerebral blood flow which is modified by oxygen and substrate demands of the tissue, and by visual stimulation and attention (Bondy, Lehman and Purdy 1974).

Amino acids are transported across cell membranes by specific carrier systems but simple diffusion is also possible.

Inside the cell the labelled amino acid becomes part of a pool of endogenous amino acid which may be the precursor for protein synthesis. The amount of radioactive amino acid incorporated will depend on the specific radioactivity of the pool, and this in turn will be determined by the amount and specific radioactivity of the precursor injected, and the quantity of endogenous, unlabelled, amino acid in the pool. Clearly both of these will vary between animals, and the latter may be subject to change during the course of an experiment. So even if protein synthesis proceeds at an identical rate in all animals, those with a higher precursor pool specific

activity would have a higher rate of incorporation. A partial answer to this is to express the results of an incorporation study as relative specific radioactivity (RSA), which is the ratio of the incorporation into proteins to the free radioactivity. This accounts for differences in incorporation generated by altered pool specific radioactivity resulting from variation in dosage.

Single, end of experiment, determinations do not however allow for changes in pool size during the experiment which might be brought about by shifts in compartmentation. Now, while protein synthesis may continue at a constant rate in an individual animal the pool specific radioactivity throughout the course of an experiment will alter, as a result of a continual dilution of the pulse of exogenous labelled amino acid as it is gradually incorporated and replaced in the pool by endogenous amino acid. Consequently, if one experimental condition involves a slower uptake into the pool, at any given time this condition will have a higher pool specific radioactivity and so a greater incorporation. Obviously, precursor pool measurements at the conclusion of an experiment do not give information about any fluctuation that may have occurred during the experiment.

Protein degradation must also be considered for over a long period labelled amino acid, once incorporated, will be returned to the pool for subsequent re-utilization. A particular experimental treatment may increase both the synthesis and breakdown of protein, which will lead to a higher turnover of labelled amino acid. In this event incorporation will increase but the amount of protein may remain the same (when synthetic and degradative rates are equal) or even decrease (if net degradation is greater).

The situation is made more complicated because the specific radioactivity of an acid soluble fraction represents not only labelled amino acids in neurones but also in glia and capillary endothelial cells, which are not the direct precursor pools for protein synthesis. Furthermore there is by no means a consensus as to whether the endogenous pool constitutes the precursor pool for protein synthesis. One view (for review of this whole question see Wheatley and Inglis 1979) suggests that amino acids entering the cell by both diffusion and active transport are immediately selected by aminoacyl tRNA synthetases for protein synthesis and the endogenous pool represents merely a discharge sump for amino acids not selected or generated by protein catabolism. In this model amino acids in the extracellular fluid serve as

the direct precursors for protein synthesis, and this is supported by the almost instantaneous and linear incorporation kinetics of essential amino acids into suspension cultured HeLa S-3 cells, which is unaffected by preloading of cells with unlabelled amino acids. Interpretation of incorporation into the acid soluble fraction is made even more problematic if this hypothesis is valid because this fraction represents several different compartments, only one of which, the extracellular fluid, is the direct precursor to protein synthesis.

Further precursor incorporation studies with the chick were conducted with such considerations in mind. Thus using radiocarbon instead of tritiated precursors prevented the problem of exchange of isotope with water and short pulse times were adopted, which not only increased the validity of precursor pool measurements but also enabled a better resolution of the time course of the process. Moreover the pulse of label was given after training had ended, which meant that all birds were in the same conditions during the incorporation period. In other words, changes in labelling would reflect previous experience rather than differences during the pulse time.

What emerged was that the increased incorporation in the anterior roof was a transient phenomenon occurring after a 60 minute exposure but not with either 30 or 120 minute training. That this was not dependent on precursor pool size was shown by a decrease in free lysine radioactivity in the anterior dorsal forebrain of exposed birds compared with dark controls. The specific activity of the free lysine was not altered by training. The elevated incorporation was found in the cytoplasmic fraction so soluble proteins were being synthesised in response to imprinting, (Hambley et al 1977).

Also because of the points raised above a short pulse time was adopted for my own incorporation experiments which gives less chance for variation in pool specific radioactivity, or re-utilization of labelled amino acid to occur (Schotman, Gipon and Gispen 1974). This means that the specific activity of the acid soluble fraction determined at the conclusion of an experiment is more likely to represent the state of the pool <u>throughout</u> the pulse.

3.5 <u>Cholinergic system studies</u>

Having established that changes occur in protein metabolism during imprinting it became important to investigate the nature of the proteins involved. Likely candidates were thought to be enzymes involved in cholinergic mechanisms. The syn-

thetic enzyme, choline acetyltransferase (CAT) showed increased activity in the midbrain immediately after the end of the exposure period; possibly a response to neurotransmitter depletion.

Several regional changes in AChE activity were identified between imprinted and dark maintained birds. One hour after a 60 minute training period the enzyme activity was 11% higher in the dorsal forebrain of trained birds. A generalised increase in AChE occurred in visually stimulated birds at 6 hours which had become a significant depression in both the dorsal forebrain and midbrain by 12 hours. Whether these alterations in AChE activity were the result of differences in activation or de novo synthesis of the enzyme is not known, although there are no in vivo co-factors affecting AChE (Haywood, Hambley and Rose 1975).

Because light control animals were not involved in this study, the results could be interpreted in terms of visual stimulation. It is known, for example, that AChE activity in the optic lobe is light dependent in young chicks (Margolis and Bondy 1969), in that 7 days after unilateral enucleation the deafferented lobe had 6% lower enzyme activity than the normal lobe. Shorter times were not studied for this measure.

TABLE 1

Biochemical	Time and direction of Modulation in dorsal forebrain (minutes)	Reference
cAMP	decrease at 15, increase by 30	Hambley and Rose (1977)
adenyl cyclase activity	increase, 60	Hambley and Rose (1977)
RNA polymerase activity	increase, 30	Haywood, Rose and Bateson (1975)
^{3}H-Uracil incorporation into RNA		
(i)		Bateson, Horn and Rose (1972)
(ii) 'split-brain'	increase, 60	Horn, Rose and Bateson (1973)
(iii) differential day 1 training	increase, 60	Bateson, Rose and Horn (1973)
^{14}C-Uracil incorporation into RNA		
(i) correlation study	increase, 120	Bateson, Horn and Rose (1975)
(ii) autoradiography	increase, 60	Horn, McCabe and Bateson (1979)
^{14}C-lysine incorporation into protein	increase, 60	Hambley et al (1977)
AIB uptake	increase, 60	Hambley et al (1977)
^{14}C-leucine incorporation into protein	increase, 60	Longstaff and Rose (in press)
^{14}C-fucose incorporation into glycoproteins	increase, 60	Dutton, Haywood and Hambley (unpublished)
AChE activity	increase, 60	Haywood, Hambley and Rose (1975)
mAChr	increase, 60	Longstaff and Rose (in press)

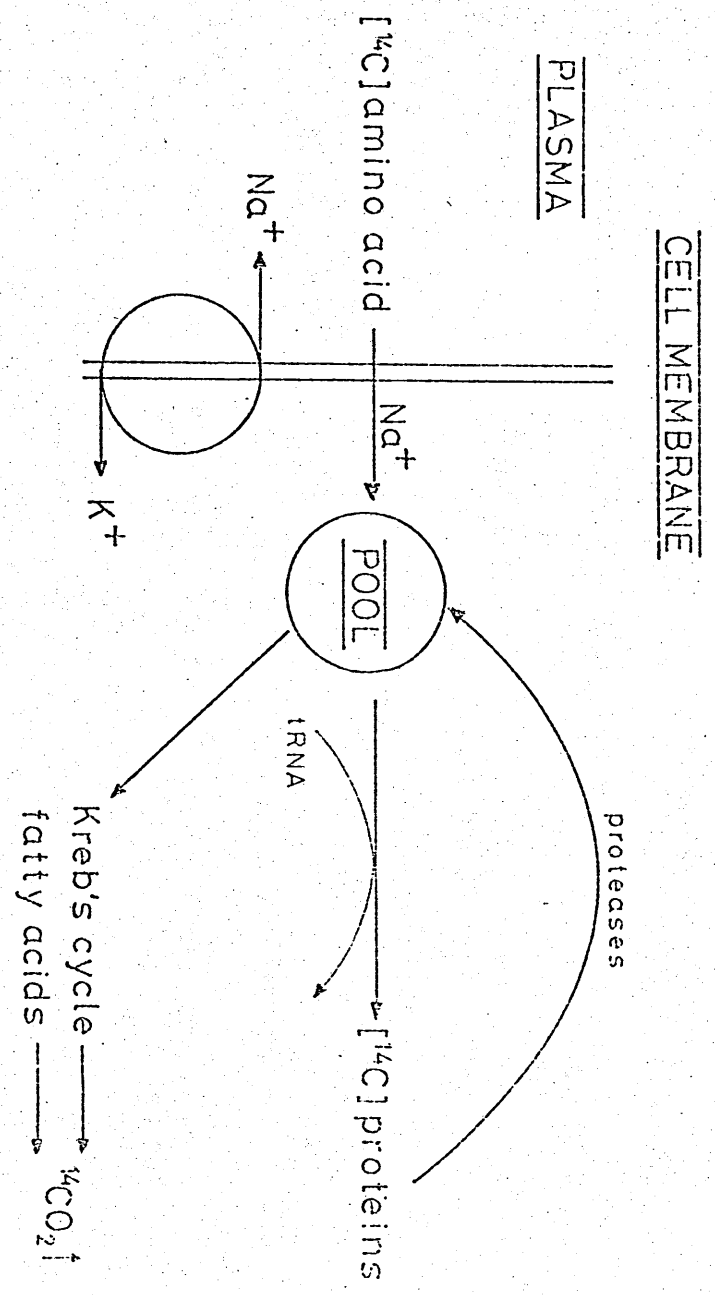

CELL MEMBRANE
PLASMA
[14C]amino acid
Na+
Na+
K+
POOL
tRNA
proteases
[14C]proteins
Kreb's cycle
fatty acids
14CO2

Fig. 1

LEGENDS

Table 1

Summary of biochemical sequelae of imprinting reported by the Brain Research Group of the Open University.

Fig. 1

Possible metabolic fate of radioactively labelled amino acids administered parenterally.

CHAPTER 4

MATERIALS AND METHODS

4.1 Training

Fertile eggs were obtained from a commercial hatchery (Hall Farm, Herringswell) and were kept at 4 C in a domestic refrigerator until the start of incubation. The strain of domestic chick (Gallus domesticus) used throughout the studies were Ross 1 (Chunky) birds, a cross between Cornish Game and New Hampshire stock developed by Ross Poultry Ltd., Sterling Division, Scotland.

For the first 18 days of incubation the eggs were maintained in darkness in a moving air incubator (Western) at 37.5 C, then transferred to a still air incubator (Curfew) at 40 C, where they hatched in the dark at 21 days from the start of incubation. Chicks which were early hatchers were removed within 4 hours. For each hatch some 70 eggs were incubated out of which normally 62-68 hatched over about 24 hours, following a skewed distribution. The first 16 birds to emerge were usually taken, although occasionally the first one or two were discarded so as to lessen the age range of the experimental birds. The time at which birds 8-10 hatched was used to calculate the start of the experiment. Upon collection the birds were housed individually in a dark brooder at 33 C until they were 21 $\pm$ 3 hours old (Plate 1). At this age the experiment began.

Chicks were coded by letter with a small self adhesive label fixed to their backs. Protocols were designed so that birds in a particular group came from throughout the hatch to minimize age effects, and received their respective treatments throughout the course of the experiment to reduce order effects.

For the majority of studies all chicks other than those which were to be exclusively dark maintained were 'primed' by exposure to diffuse light for 30 minutes, at a minimum of 30 minutes before exposure to the imprinting stimulus. For this birds were transferred to another incubator kept at the slightly lower temperature of 30 C so as to keep the animals alert. The lid of this incubator was of clear perspex covered by a sheet of white cartridge paper and illuminated by a single 60 W. tungsten lamp held vertically 50 cm above.

Birds were exposed individually for a time period which depended on the exact

nature of the experiment to one of two types of flashing light in the training apparatus. This consisted of 6 chipboard, light-tight chambers with a warm forced air flow provided by two commercial 2 kW fan heaters, each of which supplied 3 chambers, and a single extractor fan which served all chambers. A thermostatic relay (Gallenkamp) together with adjustable warm air inlets for each chamber enabled the temperature to be kept at 30 $\pm$ 1 C. The temperature was monitored during experiments using a 9 channel recorder (Grant's Instruments). A few experiments during the summer of 1976 were abandoned because the temperature could not be maintained below 35 C during training. The training apparatus is illustrated in Fig. 2 and Plate 2.

The two types of stimuli were:-

1. Red. This consisted of a transparent perspex cylinder painted with a black spiral pattern (Fig. 3).
2. Yellow. This was an opaque perspex box with the narrow sides painted black (Fig. 3).

Both were mounted onto the base of a recovery vehicle hazard lamp so that they rotated anticlockwise at 85 r.p.m. around a 48 W. bulb. Surrounding the bulb was either a red or yellow celluloid filter (Cinemoid, Strand Electric, London). Stimuli could be operated in one of 3 modes; motor on/light on; motor off/light on (Stationary stimulus) and motor on/light off, this being the normal mode for the dark chambers. The speed of rotation was adjustable with a rheostat and calibrated regularly with the aid of a stroboscope. Timers were built into the circuits of the stimuli which switched the stimuli off for one minute in every ten. This was termed 9+1 training and was adopted to limit habituation to the stimuli and keep the birds alert.

This description applies to the final design of the apparatus. For the earliest experiments the design differed:-

1. No timers were included in the circuits so the exposure was continuous.
2. The chambers were shorter so that the maximum distance of the chick from the stimulus was only 27 cm instead of the later 50 cm.
3. The yellow stimulus was somewhat larger measuring 20 x 20 x 10 cm rather than 18 x 18 x 7.5 cm.
4. For certain experiments the filter of a yellow stimulus was replaced with an orange filter.

The inside of the chambers were painted matt black to reduce reflected light.

Each chamber could accommodate 3 chicks individually housed and visually isolated from each other in hardboard pens with the ends and floor constructed of $\frac{1}{2}$ inch wire weld mesh (Plate 3). Pens were painted matt black.

Introduction of and removal of birds was always done with the stimulus turned off so that the birds could not see the experimenter. Because of rate limiting steps further on in the conduct of an experiment, such as testing or killing and dissecting, earlier manipulations of birds were staggered in accordance with a pre-arranged protocol to ensure that the time intervals between the various manipulations remained the same for all animals.

4.2 Testing

For some studies chicks were given a two choice discrimination test, which measured preference for the familiar (Bateson and Wainwright, 1972). The apparatus, which came to be termed the railway (Fig. 4 Plate 4) consisted of a wheel of diameter 30 cm, with black perspex sides and a wire mesh circumference, which was free to move along a 220 cm metal track. At each end of the track was a flashing light, at one end red and at the other yellow, of the same design as those which had served as stimuli during training. The gearing of the wheel is such that when the chick, which is placed in the wheel through a trap door in the wire mesh, attempts to move towards the stimulus of its choice it is carried in the reverse direction. Further, the gearing ratio is nearly 5:1 so that a bird walking the equivalent of 100 cm is taken only 18 cm by the wheel. The friction in the system is low and a 50 g day old chick has no difficulty in moving the wheel.

Various measures using the railway have been tried, but the only one which correlated significantly with the previous imprinting experience of the bird was the maximum distance moved from the midpoint of the track in any direction during the course of the test. This was termed the preference score (Bateson and Wainwright, 1972).

Since larger stimuli are more effective in eliciting approach behaviour (Fabricus and Boyd, 1954; Smith and Hoyes; 1961) were the chick to be carried in the direction of its choice the stimulus would appear larger and so be more likely than the alternative stimulus to generate such behaviour. The chick would be 'trapped' by its first movements, which are probably only exploratory, since the animal is unused to the sensation in the wheel, having previously been trained in a stationary pen.

The reverse gearing overcomes this problem and the test really becomes one of determining how close (i.e. large) the novel stimulus has to be before it competes equally with the familiar stimulus in eliciting approach.

In practice however during the course of a 4 minute test chicks do not take up an equilibrium position this notion would predict, but oscillate back and forth depending on which stimulus they happen to be attending to at that particular moment. With the wide peripheral vision which birds have, it is possible for a chick to sit facing the side of the wheel and attend to both stimuli simultaneously, and while birds frequently did this, especially at the beginning of the test, they had to turn to face one of the stimuli in order to propel the wheel. Although the chicks were carried in the direction opposite to their choice, negative reinforcement for the familiar stimulus was thought unlikely because of the short time involved, and because animals left in the railway will eventually learn to run backwards in order to approach the familiar stimulus (Pat Bateson, Personal Communication). While chicks frequently emitted distress calls throughout the test this may have been a response to the low temperature (27 $\pm$ 2 C).

Tests were conducted in the following way. Chicks were introduced into the wheel, which was initially located at the midpoint of the railway, either feet or head first. With the experimental protocol used, half of the chicks trained on any particular stimulus would start off facing one way, and half the other. Simultaneously starting a digital timer and switching on the stimuli marked the beginning of the test. At the end of each minute the position of the wheel on the track was recorded with a convention adopted that movement indicating preference for the familiar would score as positive, preference for novelty as negative. Throughout the test, each time the wheel changed direction the position was noted, so that it was possible to calculate the total distance moved by the wheel in all directions, giving a crude activity score. The preference score (see above) was the key measure and because of the construction of the railway actually ranged from +104 to -104 cm. At the end of the test the stimuli were switched off and the bird removed with the aid of a dim green light.

4.3 Injection of radiolabelled precursors

Most of the experiments involved injecting animals with radiolabelled amino acids. These were given as a 0.1 ml aliquot with a gauge 26 needle inserted full length (10 mm) immediately caudal to the sternum in the midline and directed upwards

into the thorax at an angle of about 45°. Much of the time this did succeed in introducing the solution directly into the heart since:

1. A backsurge of blood was frequently noted at the end of the injection.
2. On necropsy following the injection of methylene blue, dye was often found to have stained the endocardium of the ventricles but could not be seen external to the heart, except for a small amount of staining along the needle track.
3. Hemopericardium was often noted following occasional necropsy of experimental birds.

However the dye studies frequently revealed material deposited around the heart and for this reason the term pericardial is preferred to describe the injection route. The technique always succeeded in avoiding the yolk sac, and despite occasionally finding blood clots at necropsy, the safety of the procedure is attested by the low mortality; in my hands 1 chick died out of a total of 514 (0.002%) over the 20 minute period after injection. Injections took between 10-20 seconds, during which time the chick was firmly but gently restrained so that it could not move its body or wings. Birds often emitted distress calls but this seemed to be related to the handling rather than the injection itself. Observations of birds after the injection revealed no obvious alterations in behaviour.

A pericardial route probably permits a more rapid cerebral uptake of precursor than the subcutaneous method, which is important for short pulse times in order to avoid changes in pool specific activity over the immediate post-injection period. An intraperitoneal route risks sequestering material in the yolk sac which markedly reduces uptake of radioactivity by the brain. An intracranial injection, quite apart from producing unnecessary damage to the brain, seems to produce increased drowsiness, even when done without anaesthetic. The pericardial route appears to cause no more stress to the chicks than other routes.

4.4 Killing

Chicks were killed by one of two methods depending on the biochemical nature of the experiment.

1. For those experiments which were confined to radiolabelled precursor incorporation studies the animals were subjected to microwave radiation (2450 MHz, 1100 W) in a commercial microwave oven for 5 seconds. This rapid method of halting metabolism by heat denaturation of proteins is particularly important when short pulse times are used. Since the dissection was found to be more difficult,

when the tissue was still warm, the birds were placed in a freezer at -20 C for about 30 minutes following irradiation.

2. For all other experiments the birds were decapitated with a single cut by a Mayo's scissors.

4.5 Brain dissection

It was felt necessary, at least initially to be able to relate experiments to previous work and for this reason the dissection scheme was adopted essentially unchanged.

The brain was removed and the hindbrain including the cerebellum discarded. A cut immediately anterior to the posterior commissure separated off the midbrain including the optic lobes. The remaining forebrain was inverted into an araldite mould (the same mould being used throughout) so that the base of the forebrain projected above the surface and could be divided from the roof by a single left to right sweep of a scalpel blade (Swann Morton No 25) held horizontally, and with the tip of the handle acting as a brace and guide along the edge of the mould. The forebrain base contains much of the thalamus, neostriatum, paleostriatum and archistriatum which includes the hippocampus. The forebrain roof consisting largely of hyperstriatum was then removed from the mould and dorsal side uppermost dissected into an anterior and a posterior part. Brain regions dissected in this way from different birds having the same body weights did not differ in weight by $\pm$ 10%. The dissection is illustrated in Fig. 5 and Plates 5 a-d.

4.6 Incorporation into all proteins

The earliest radiolabelled protein precursor incorporation studies used (^{14}C) L-lysine because this amino acid is not markedly catabolised. The later experiments in which the synthesis of acidic proteins such as microtubule protein were of interest used (^{14}C) L-leucine, since tubulin has a much higher leucine than lysine content. Tritium radiolabelled amino acids were not used because of the problems associated with the exchange of tritium with water.

For the lysine incorporation experiments the following procedures were adopted. Each brain sample was homogenised in 5 ml of 155 mM NaCl with 16 strokes of a glass-teflon homogeniser, clearance 200-250 μm, to give a protein concentration of 1-4 mg ml^{-1}. A 2 ml aliquot of the homogenate was precipitated with 10 ml of 10% w/v trichloroacetic acid (TCA) plus 10 mM lysine at 4 C. The precipitate was spun down at 1,000 x g for 10 minutes (Mistral 4L centrifuge, MSE) and twice

washed with further 10 ml aliquots of this TCA solution. The washed precipitates were digested with 0.3-0.5 ml Nuclear Chicago Solubilizer (NCS, Amersham-Searle), the quantity added depending on the protein concentration, at 60 C on a heating block (Dri-Block, Techni Instruments, Cambridge). When dissolution was complete the digest was washed into a glass scintillation vial with two 5 ml aliquots of a scintillation cocktail containing 1:1 v/v toluene and methoxyethanol, 6 g l^{-1} 2, 5-diphenyloxazole (PPO) and 0.6 g l^{-1} dimethyl P bis 2-(5 phenyloxazolyl)-benzene (POPOP). This was counted in a liquid scintillation spectrophotometer (Beckman LS 150 or LS 250) at an efficiency of 70-75% with an error of 3-5%. The counts were corrected for background radiation but since the external standard ratios (ESR) were within 3-5% no quench correction was warranted. This gave a measure of the lysine incorporated into proteins. Total counts were obtained by digesting 1 ml of the homogenate in 0.2-0.3 ml NCS, solubilizing in scintillant and counting as above. The free radioactivity was calculated by difference between the total and acid insoluble counts. The results were expressed as specific radioactivities (cpm mg^{-1} protein).

With the leucine incorporation studies some modifications were made to the above methods. Each brain region was homogenised with a polytron (Kinematica, Switzerland) in 5 ml of a buffer containing 100 mM sodium glutamate and 20 mM disodium hydrogen phosphate at a pH 6.8 to give a protein concentration of 1-4 mg ml^{-1}. A 1 ml aliquot of this homogenate was precipitated and twice washed with 10% w/v TCA + 10 mM leucine at 4 C, digested with 0.3 ml NCS and solubilized in a scintillation fluid containing 1:1 v/v toluene and methoxyethanol, 6 g l^{-1} PPO and 1% w/v Triton X-100.

Total counts were obtained by digesting 0.5 ml of the homogenate in 0.2 ml NCS and adding 10 ml of the scintillation cocktail. Samples were counted as above and the results expressed as standardised specific radioactivities (cpm mg^{-1} protein).

4.7 Leucine metabolism

The metabolic fate of leucine may include deamination, (indeed L-amino acid oxidase activity has been detected in avian liver) or transamination with oxoglutarate as the amino-acceptor, both of which generate the corresponding keto-acid, α-oxoisocaproate. Further catalysis of this metabolite yields both acetoacetic acid and acetyl-SCoA. Since this intermediate is the key precursor for fatty acid biosynthesis, and trichloracetic acid precipitates the lipids in homo-

genates, an investigation of the extent to which leucine derived radioactivity is incorporated into fats was undertaken.

5 μCi of (U-^{14}C) L-leucine were injected into 9 birds which were killed by decapitation after 20 minutes. The forebrains were dissected out and worked up separately. Each was macerated with a top drive macerator in 10 ml of chloroform/methanol, 2:1 v/v. Pilot experiments had attempted to homogenize the tissue but failed to produce a homogenous mixture in organic solvents. 100 μl aliquots of the homogenate were taken and counted for total radioactivity to ascertain the recovery of radioactivity. The homogenate was spun at 1,000 x g for 10 minutes (Mistral 4L centrifuge, MSE). The pellet containing the protein was washed twice, then digested in NCS at 80 C on a heating block. To the combined supernatants 0.2 vols. of 0.05 M NaCl were added; it was then shaken and centrifuged at 1,000 x g for 20 minutes to effect separation into an upper layer containing water and methanol, and a lower layer containing chloroform with dissolved lipids. The upper layer was removed with a pasteur pipette, and the lower layer washed 3 times with chloroform/methanol/water, 3:47:48 v/v. The rinsing interface was dispelled by the addition of approximately 1-2 ml methanol. The lower layer was evaporated to dryness overnight at 80 C and re-extracted with chloroform/methanol, 2:1. A 1,000 x g spin for 10 minutes of this re-extracted lower layer separated an insoluble proteolipid precipitate from the lipid supernatant. This precipitate was so slight however that its contribution to the radioactivity was not determined independently of the supernatant. The procedure described above is essentially that formulated by Folch, Lees and Sloane Stanley (1956). All of the samples, both pellet and supernatant were counted by liquid scintillation spectrophotometry in Bray's cocktail at an efficiency of 70%. The counts were corrected for background radiation. The results are expressed as means $\pm$ SEM of the total counts (cpm) from both the protein and lipid fractions. The mean recovery of radioactivity was 92% and a flow diagram of the extraction procedure is given in Fig. 6.

Protein fraction	lipid fractions
29924 $\pm$ 3851	1000 $\pm$ 134

This indicates that only 3.34 $\pm$ 0.5% of the radioactivity injected as leucine was recoverable from the lipid fraction.

4.8 Incorporation into acidic protein fraction

Acidic protein fractions were prepared by absorbing 100 μl aliquots of homogenate containing 100-300 μg protein onto a stack of 4 DEAE cellulose discs (Whatman, DE 81). The discs were left for 10 minutes and then washed 4 times with 5 ml portions of 20 mM sodium phosphate buffer pH 6.8 at 4 C, under vacuum, using a filter manifold (Millipore, USA). The discs were counted in a Bray's scintillation cocktail containing 8 g l^{-1} PPO, 0.6 g l^{-1} POPOP, 150 g l^{-1} naphthalene, 10% v/v ethoxyethanol, and made excipient to one liter with 1.4-dioxan; at an efficiency of 70% in a liquid scintillation spectrophotometer (Beckman LS 250). The counts were corrected for background radiation but no quench correction was required nor applied. Initially samples were estimated in triplicate but as reproducibility was found to be within 5% later samples were only assayed in duplicate. The results were expressed as standardised specific radioactivities (cpm mg^{-1} protein).

4.9 Colchicine binding assay

The tubulin content of brain samples was estimated by a colchicine binding assay (Weisenberg, Borisy and Taylor 1968). 200 μl aliquots of chick brain homogenised in sodium glutamate buffer (see above) were incubated at 37 C for one hour with (Ring C-methoxy ^{3}H) colchicine, specific radioactivity 5.9 Ci $mmole^{-1}$ (Amersham Radiochemical Centre) so that the final concentration of the alkaloid including cold carrier was 2.5 x 10^{-6} M. Each sample was assayed in triplicate. 100 μl aliquots of the incubates were placed onto a stack of 4 DEAE cellulose discs moistened with 20 mM sodium phosphate buffer at pH 6.8, washed under vacuum with this buffer 5 times to remove unbound colchicine, and counted in Bray's scintillation cocktail at an efficiency of 20%. Counts were corrected for background radiation and from quench curves which plotted the external standard ratio (ESR) against the percentage efficiency of counting, a quench correction was applied. The results were expressed as standardised specific radioactivities (dpm mg^{-1} protein). A number of pilot studies were undertaken on fresh tissue to determine the characteristics and reproducibility of the assay and a description of these together with their findings is given in Figs. 7-10.

4.10 Acetylcholinestenase (AChE) assay

The activity of AChE was estimated by a modification of the method of Ellman et al (1961). For the earlier experiments and the pilot studies the assays were done manually, later experiments used an automated method which will be

described subsequently.

The method is based on the hydrolysis of the artificial substrate acetylthiocholine iodide (ATC). Liberated thiocholine reacts with the colour reagent dithiobisnitrobenzoate (DTNB) to produce the yellow coloured 5-thio-2-nitrobenzoid acid, which can be determined spectrophotometrically by its absorption at 412 nm.

For the manual method, 100 μl of chick brain homogenized in sodium glutamate buffer (see above) were suspended in 100 mM Tris buffer at pH 8.0 containing 0.3 mM dithiobisnitrobenzoate (DTNB) with 0.5 mM acetylthiocholine iodide as substrate and 300 μM tetraisopropyl pyrophosphoramide as an inhibitor of butyrylcholinesterase. This mixture was incubated at 37 C for 20 minutes at the end of which the tubes were placed on ice and the optical density at 412 nm was recorded using a flow cell spectrophotometer, as soon as possible after the conclusion of the incubation. Each sample was estimated in triplicate, and where agreement between the replicates was not within 10% the assay was repeated. Assays were done in batches of 18, and with each batch controls were run in which tissue was omitted from the incubation medium, so that non-enzymic hydrolysis of the substrate could be corrected for. The results are expressed as standardised specific activities (mkat kg^{-1} protein). Pilot studies used to establish appropriate conditions for the assay are summarized in Figs. 12-14.

An automated method for assaying AChE was developed using an autoanalyser (Technicon NC2P). The system (Fig. 11) comprised of an automatic sampler set to sample at the rate of 40 per hour. The best reproducibility was obtained when a sample to wash ratio of 1:1 was employed, and when the wash contained 50% aqueous ethanol plus 0.1% Triton X-100. The actual rate of tissue sampling then became 20 per hour. Reagents prepared fresh at the beginning of each week were delivered by a single speed 26 channel proportioning pump at the flow rates annotated to give the same final concentrations in the assay mix that were used for the manual method.

Samples diluted 20 fold in glutamate buffer were separated by the injection of air bubbles into the flow line and mixing coils were incorporated into the line whenever a reagent was added. Since the samples were crude homogenates, the artificial substrate was the last reagent to be added; permitting a short pre- incubation of

the tissue and thus enabling catalysis of endogenous acetylcholine. Incubation proceeded at 37 C for 9 minutes. The optical density was determined at 412 ± 1 nm using a spectrophotometer with a photometric range 0.05-2.0 absorbance units, quoted accuracy of 1%, fitted with a 15 mm path length flow cell, and recorded on a linear 2 channel 60 mV servoscribe recorder (AA 1) with the base-line adjusted to the absorbance given by the glutamate homogenizing buffer. The total flow-through time was 11.5 minutes.

The output from the spectrophotometer was calibrated daily by the use of 5 standard solutions of bovine erythrocyte acetylcholinesterase with activity of 2.6 units mg^{-1} (Sigma, Type I). Once calibrated the system was stable throughout the day. All samples were run in duplicate and agreement between replicates was within 1%.

4.11 Estimation of muscarinic receptor (mAChr)

mAChr was assayed by atropine displaceable binding of the muscarinic antagonist ^{3}H-Quinuclidinyl benzilate (QNB). The method was essentially that developed by Yamamura and Snyder (1974). 100 µl aliquots of chick brain originally homogenised in sodium glutamate buffer was suspended in a buffer containing 0.05 M sodium, potassium phosphate (pH 7.4) and ^{3}H-QNB with a specific radioactivity of 8.4 Ci $mmole^{-1}$ (Amersham Radiochemical Centre) was added to give a final concentration in the assay mix of 6 nM. In order to determine the degree of specific binding of the ligand to mAChr all samples were assayed both in the presence and absence of 12.5 µM atropine sulphate. Each sample was incubated in triplicate both with and without the alkaloid. Following a one hour incubation at 25 C the assay mixtures were filtered through 2.5 cm diameter glass fibre discs (Whatman GF B), under vacuum, using a filter manifold (Millipore, USA). Each disc was washed 5 times with 2 ml of the 0.05 M phosphate buffer at 4 C to remove unbound radioactivity, and placed in a scintillation vial with 8 ml of a cocktail containing 1:1 v/v toluene and methoxyethanol, 6 g l^{-1} PPO and 1% v/v Triton X-100. The samples were counted in a liquid scintillation spectrophotometer (Beckman LS 150) at an efficiency of 20%. The counts obtained were corrected for background radiation and quenching and expressed as standardised specific activities (fmoles QNB bound mg^{-1} protein). Pilot studies used to establish appropriate conditions for the assay are summarized in Figs. 15-17.

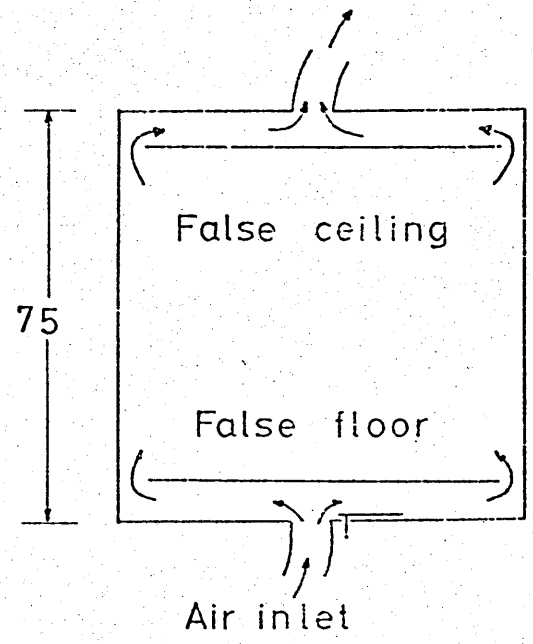
4.12 Protein estimation

Protein concentration was estimated by the method of Lowry et al (1951) using bovine serum albumin as a standard. 100 μl aliquots were used containing 100-400 μg protein; over which range the assay was linear. Where aliquots contained more than 400 μg protein they were diluted to avoid problems of non-linearity. The optical density at 500 nm was determined using a spectrophotometer, fitted with a flow cell. Each sample was routinely run in duplicate and agreement between replicates usually fell within 3%.

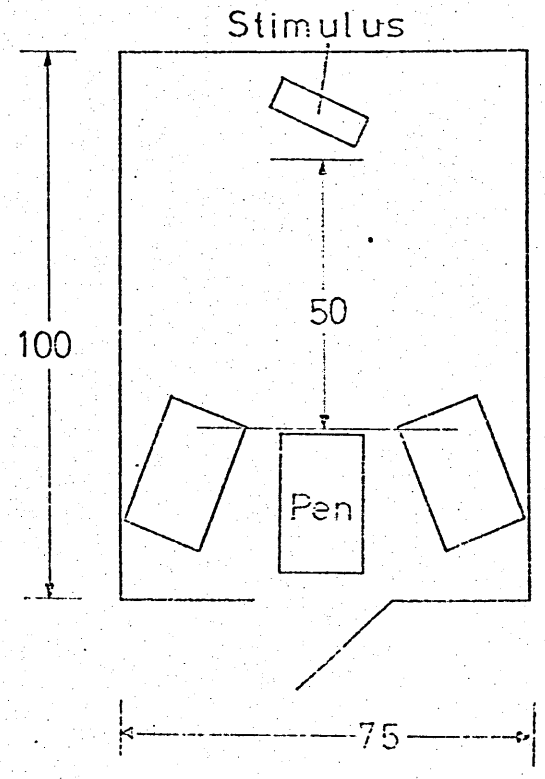
4.13 Standardization and statistical methods

Considerable interhatch variation has been described for the chick (see e.g. Bateson, Horn and Rose, 1975; Benowitz and Shashoua 1979) and was anticipated for the experiments described here. To reduce this variation a standardization procedure was adopted in which the raw biochemical measures obtained for an individual bird was multiplied by the ratio of the mean value for all birds in the same hatch to the mean for birds in all hatches. The experimental design was balanced in that all groups were similarly represented in each hatch, and each hatch contained an equal number of birds.

Where statistical methods have been used they are defined. All such manipulations were done with either HP 65, HP 97 (Hewlitt Packard) or TI 59 (Texas Instruments) programmable calculators, using statistics software provided by the manufacturers.



T.S.SECTION



PLAN

Fig. 2

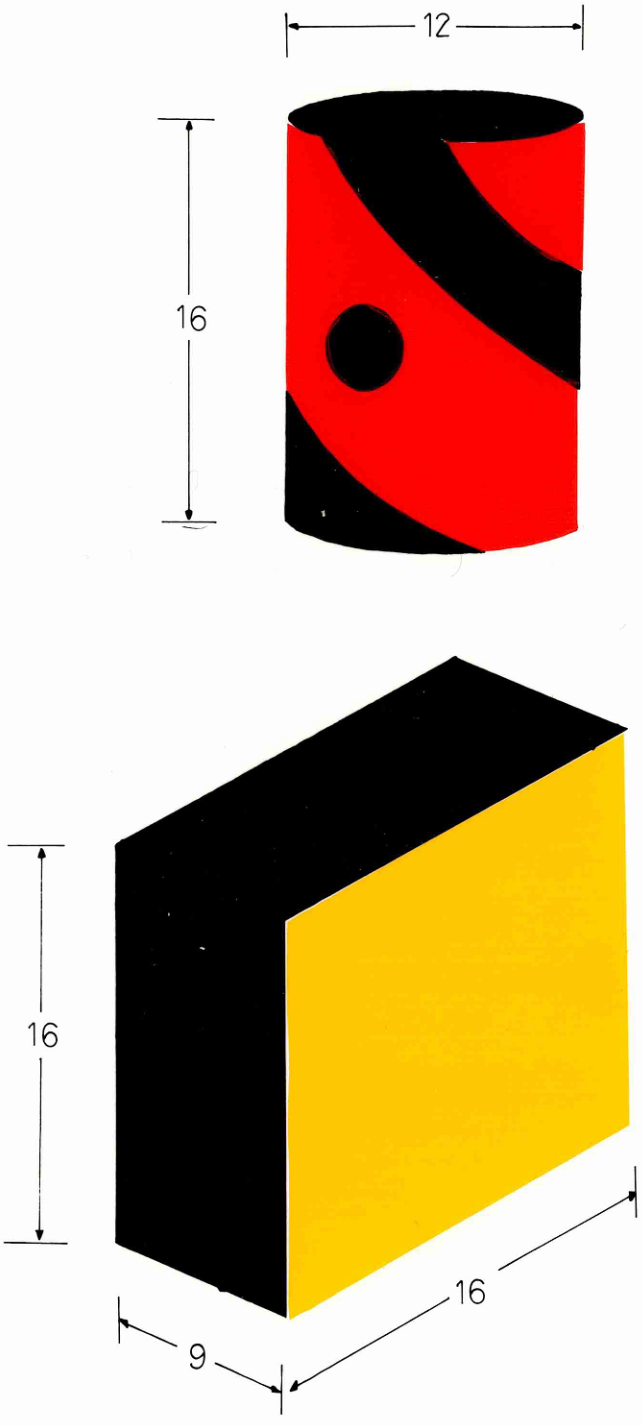


Fig 3

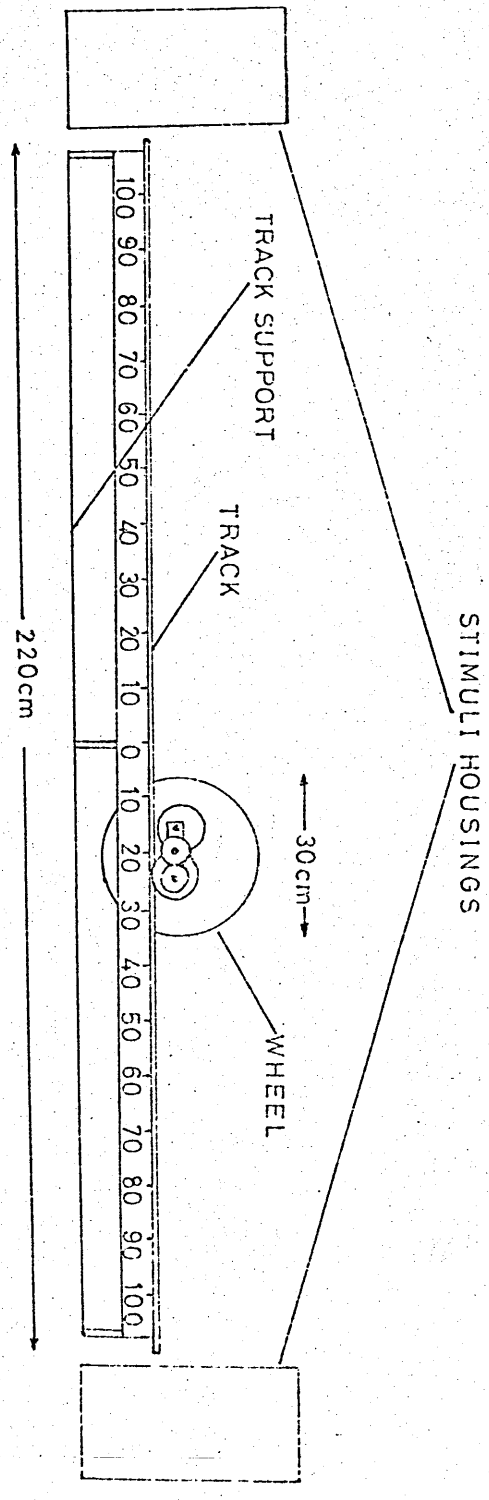

TRACK SUPPORT
TRACK
STIMULI HOUSINGS
220cm
30cm
WHEEL
100 90 80 70 60 50 40 30 20 10 0 10 20 30 40 50 60 70 80 90 100

Fig. 4

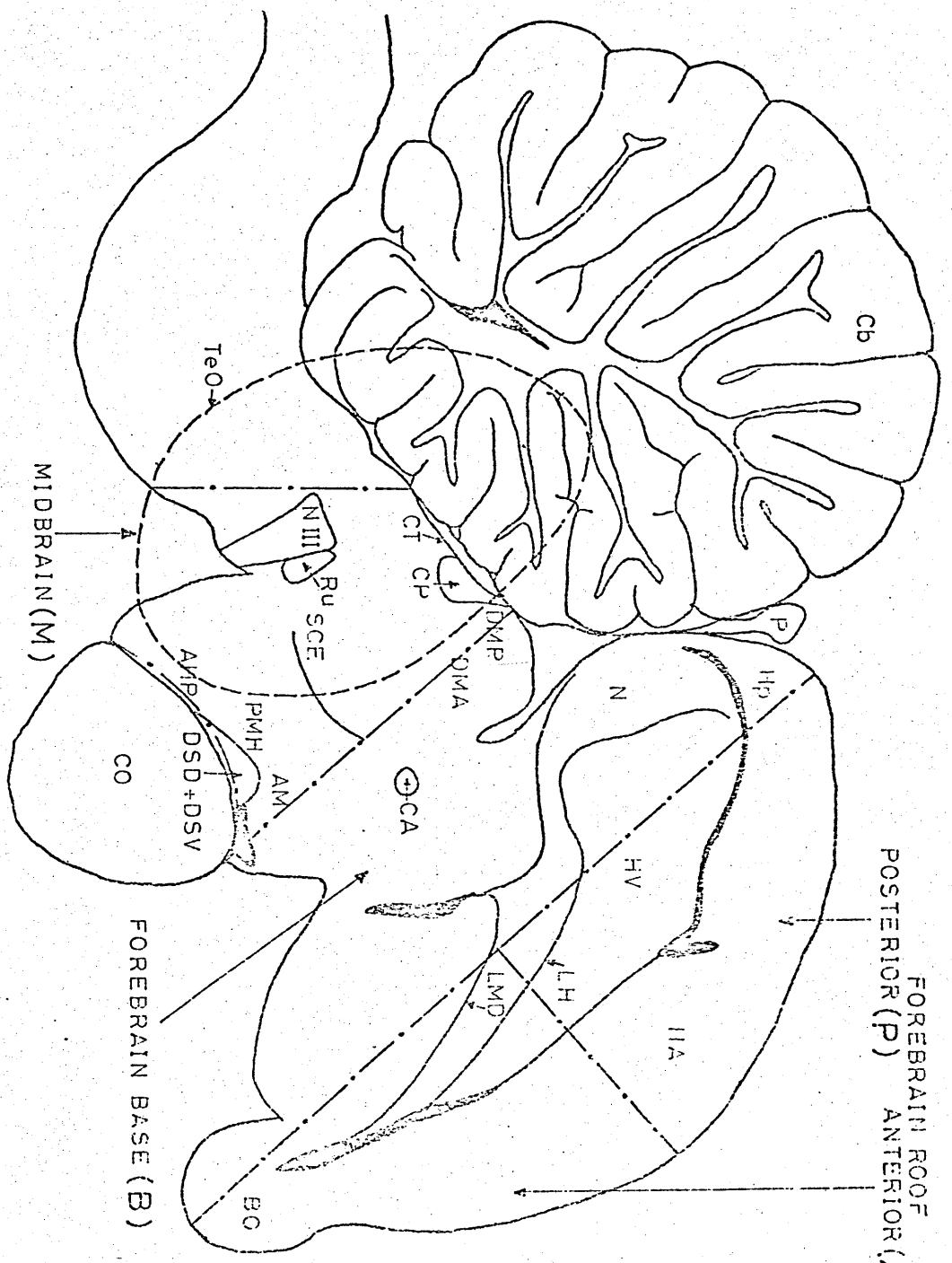
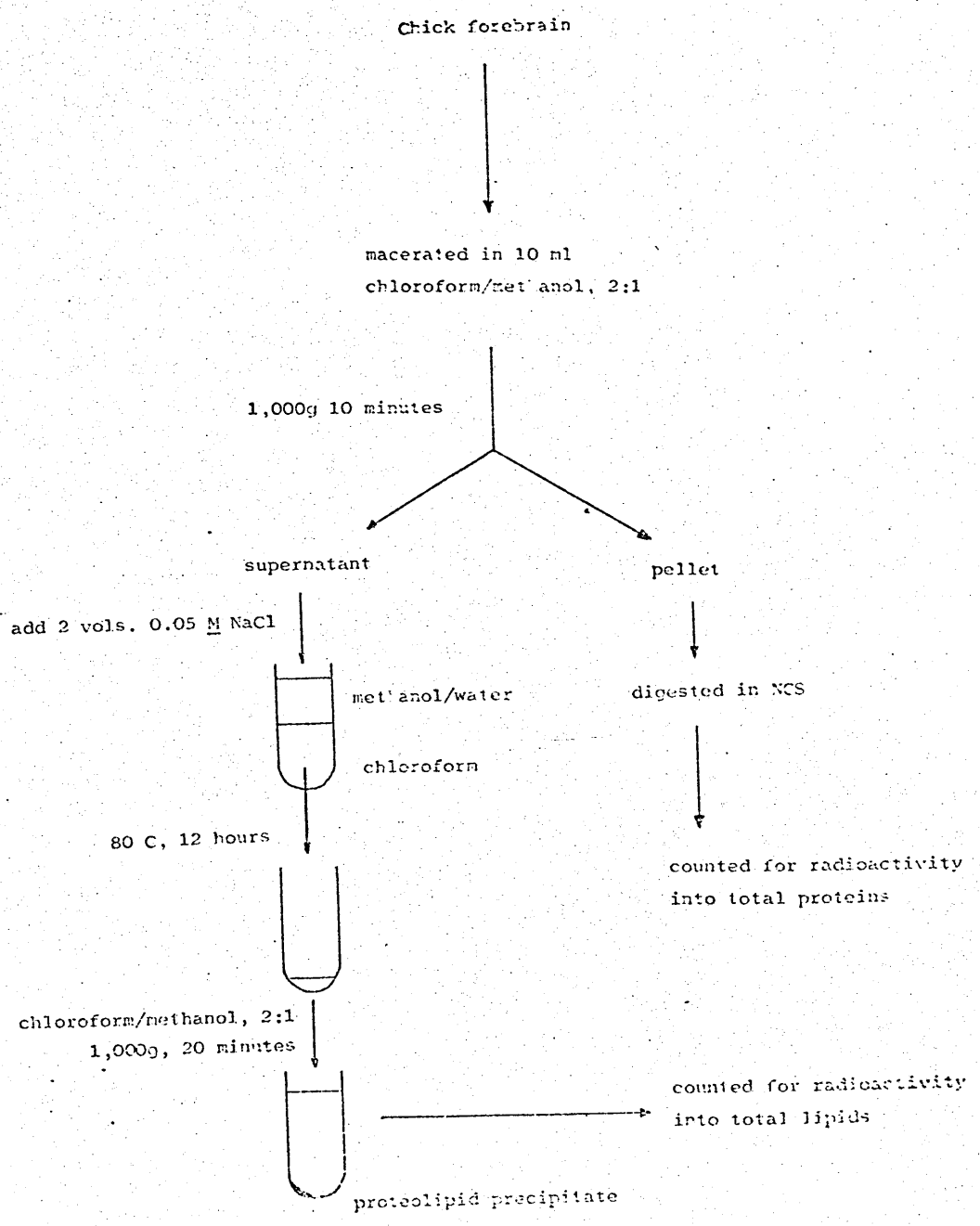
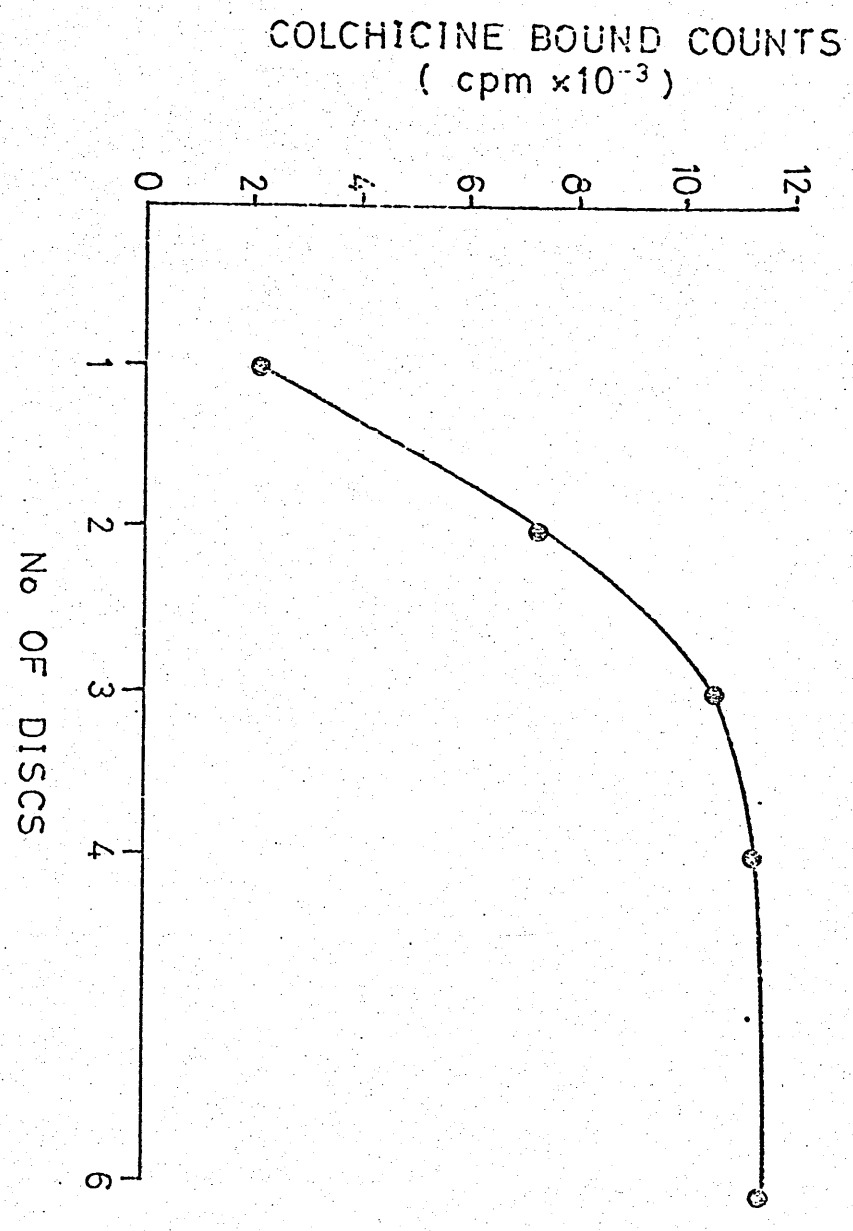

Cb
TeO
MIDBRAIN (M)
NIII
Ru
SCE
CT
CP
DMP
DMA
P
Hp
N
HV
LH
LMD
IIA
BO
FOREBRAIN ROOF
POSTERIOR (P)
ANTERIOR (A)
AVP
PMH
DSD+DSV
AM
CA
CO
FOREBRAIN BASE (B)

Fig. 5



For clarity wash steps are not included in this flow diagram but are described in the text.

Fig. 6


COLCHICINE BOUND COUNTS
(cpm ×10⁻³)
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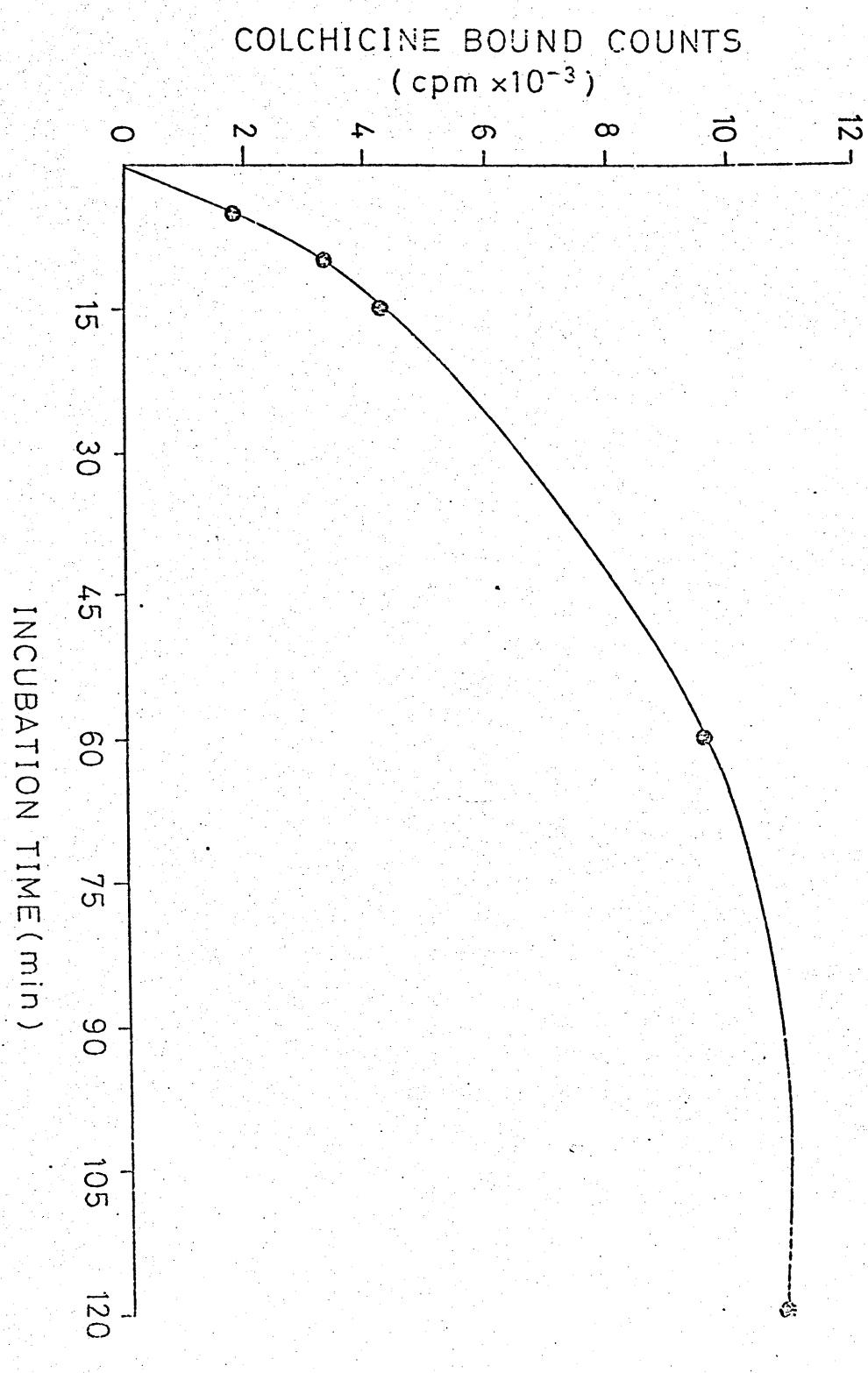
2
4
6
8
10
12
No OF DISCS
1
2
3
4
6

Fig. 7


COLCHICINE BOUND COUNTS
(cpm ×10⁻³)
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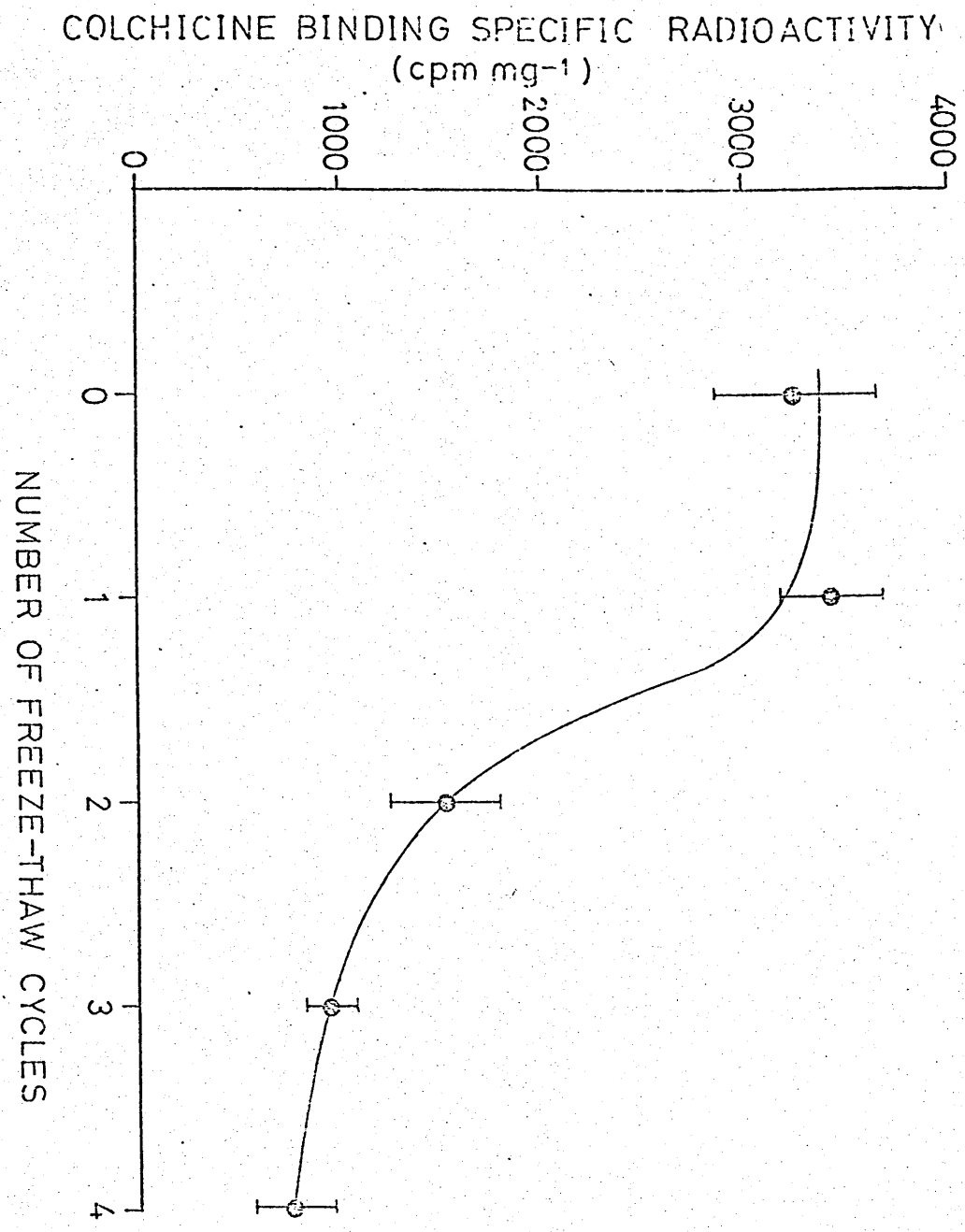
2
4
6
8
10
12
INCUBATION TIME (min)
15
30
45
60
75
90
105
120

Fig. 8


COLCHICINE BINDING SPECIFIC RADIOACTIVITY
(cpm mg-1)
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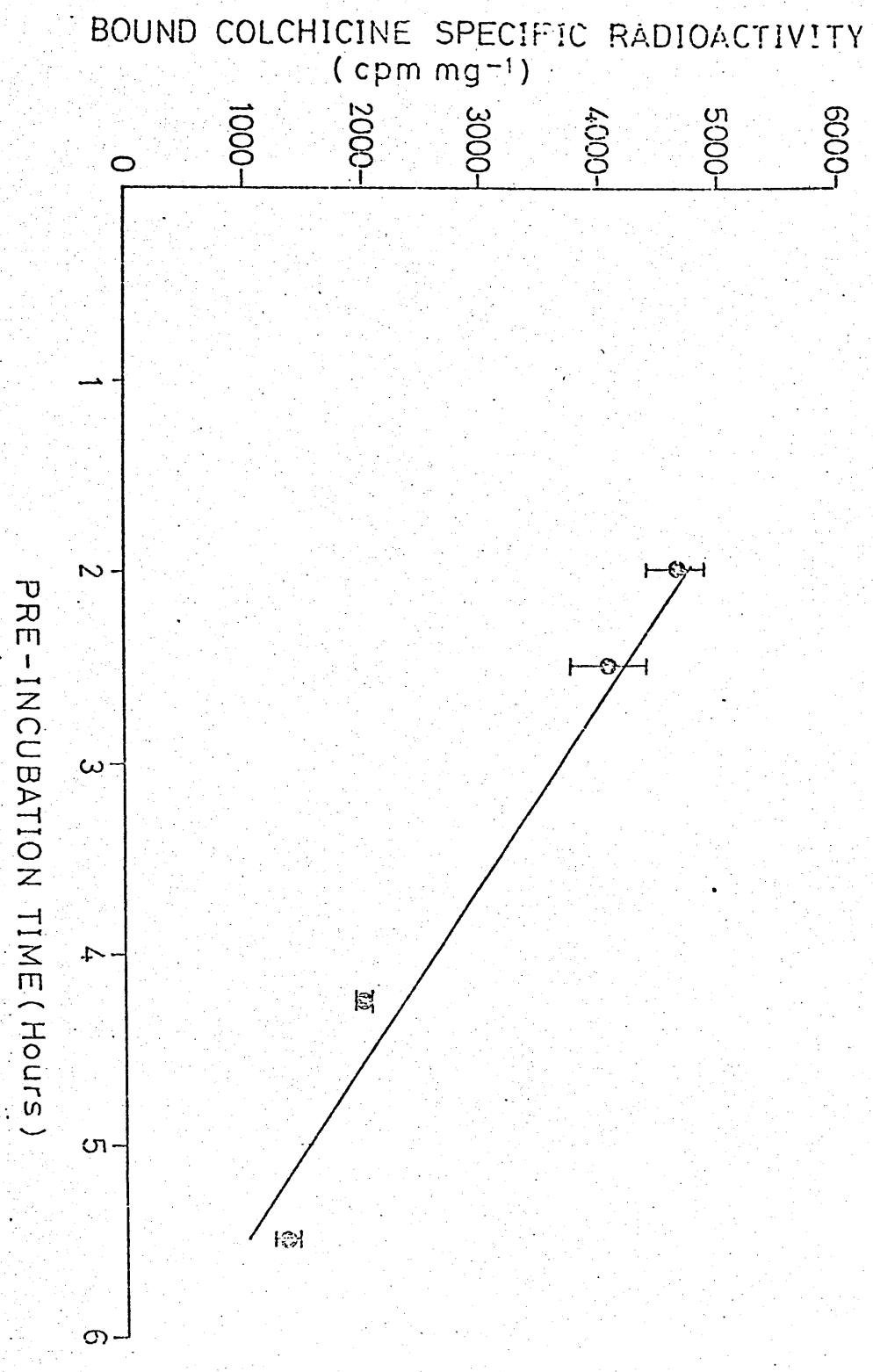
3000
4000
NUMBER OF FREEZE-THAW CYCLES
0
1
2
3
4

Fig. 9


BOUND COLCHICINE SPECIFIC RADIOACTIVITY
(cpm mg⁻¹)
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1000
2000
3000
4000
5000
6000
PRE-INCUBATION TIME (Hours)
1
2
3
4
5
6

Fig. 10

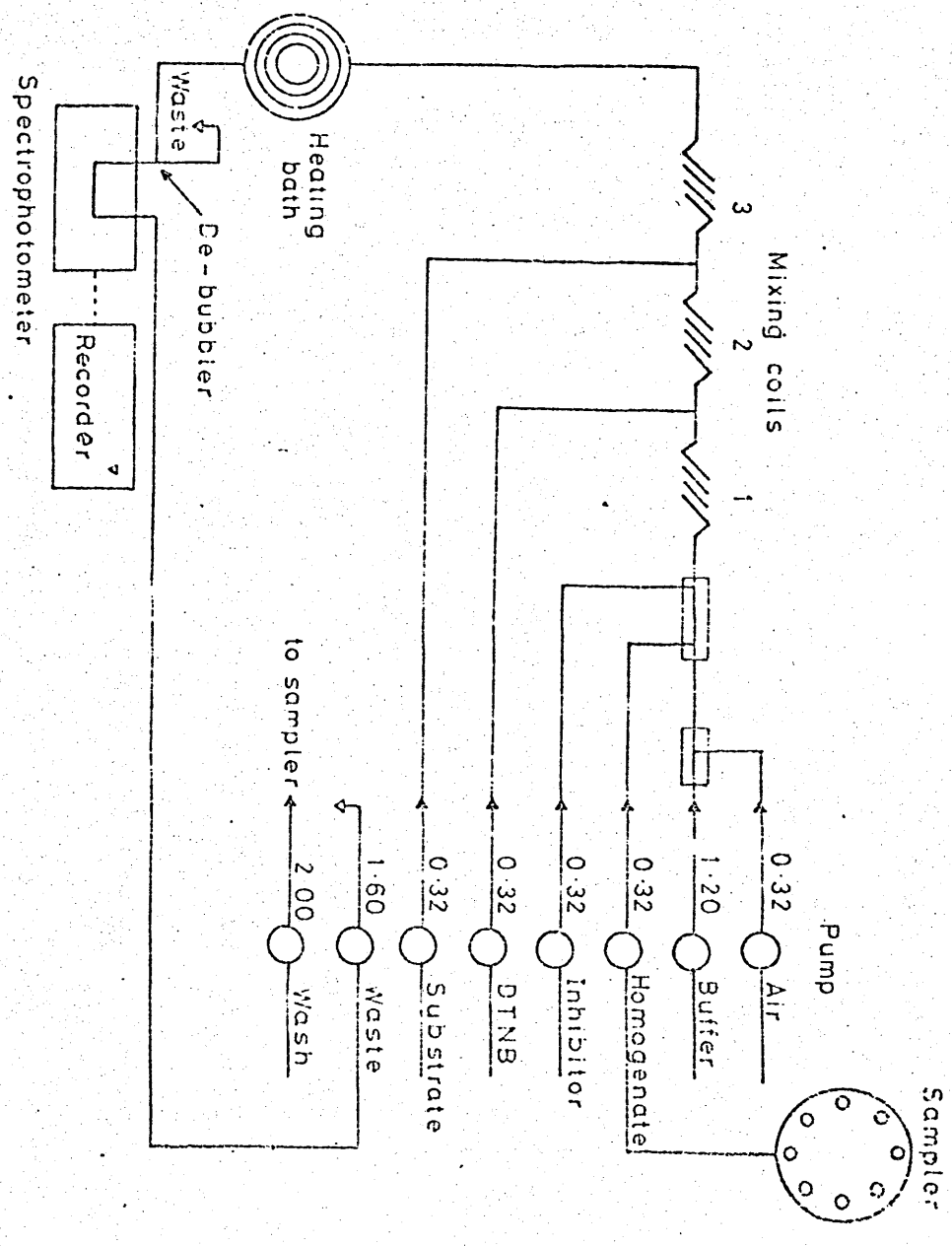

Sampler
Pump
Air
0·32
Buffer
1·20
Homogenate
0·32
Inhibitor
0·32
DTNB
0·32
Substrate
0·32
Waste
1·60
Wash
2·00
to sampler
Mixing coils
3
2
1
Heating bath
Waste
De-bubbler
Spectrophotometer
Recorder

Fig. 11

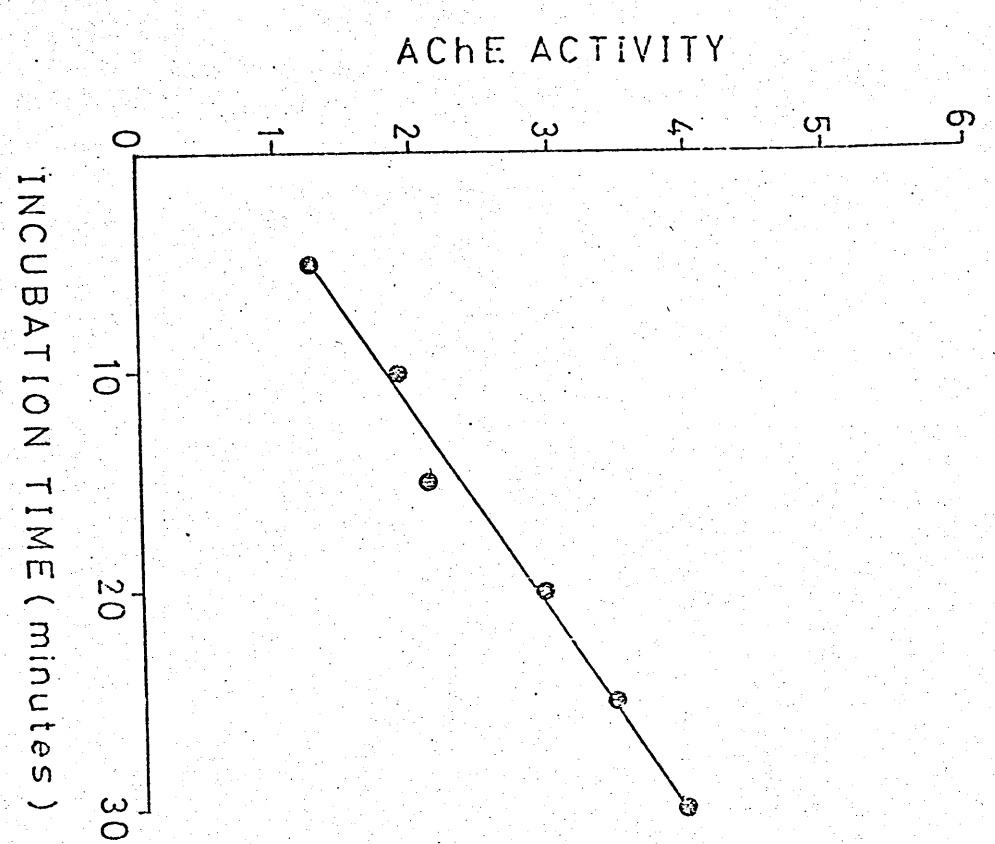


Fig. 12

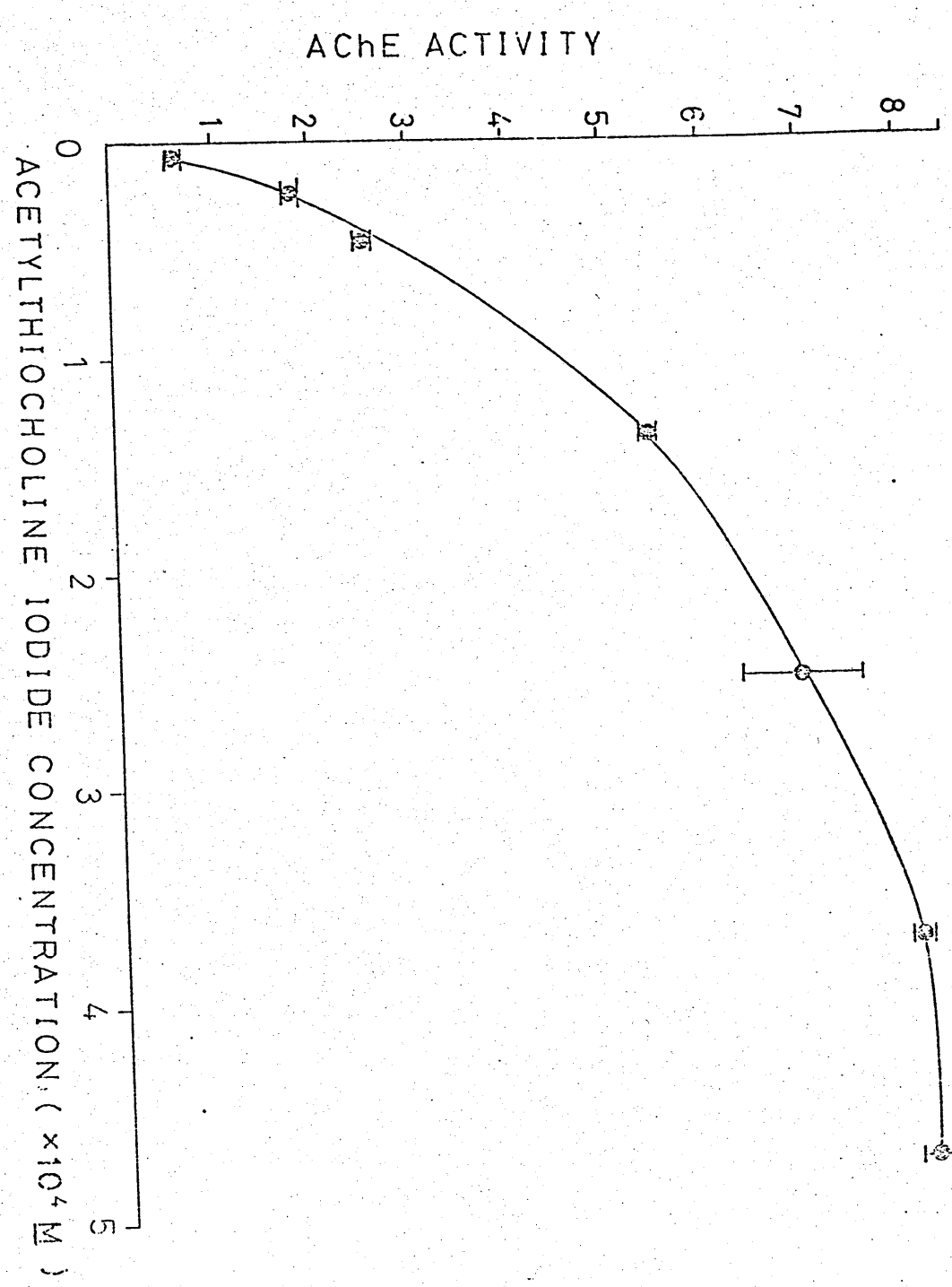

AChE ACTIVITY
1
2
3
4
5
6
7
8
0
1
2
3
4
5
ACETYLTHIOCHOLINE IODIDE CONCENTRATION (×10⁴ M)

Fig. 13

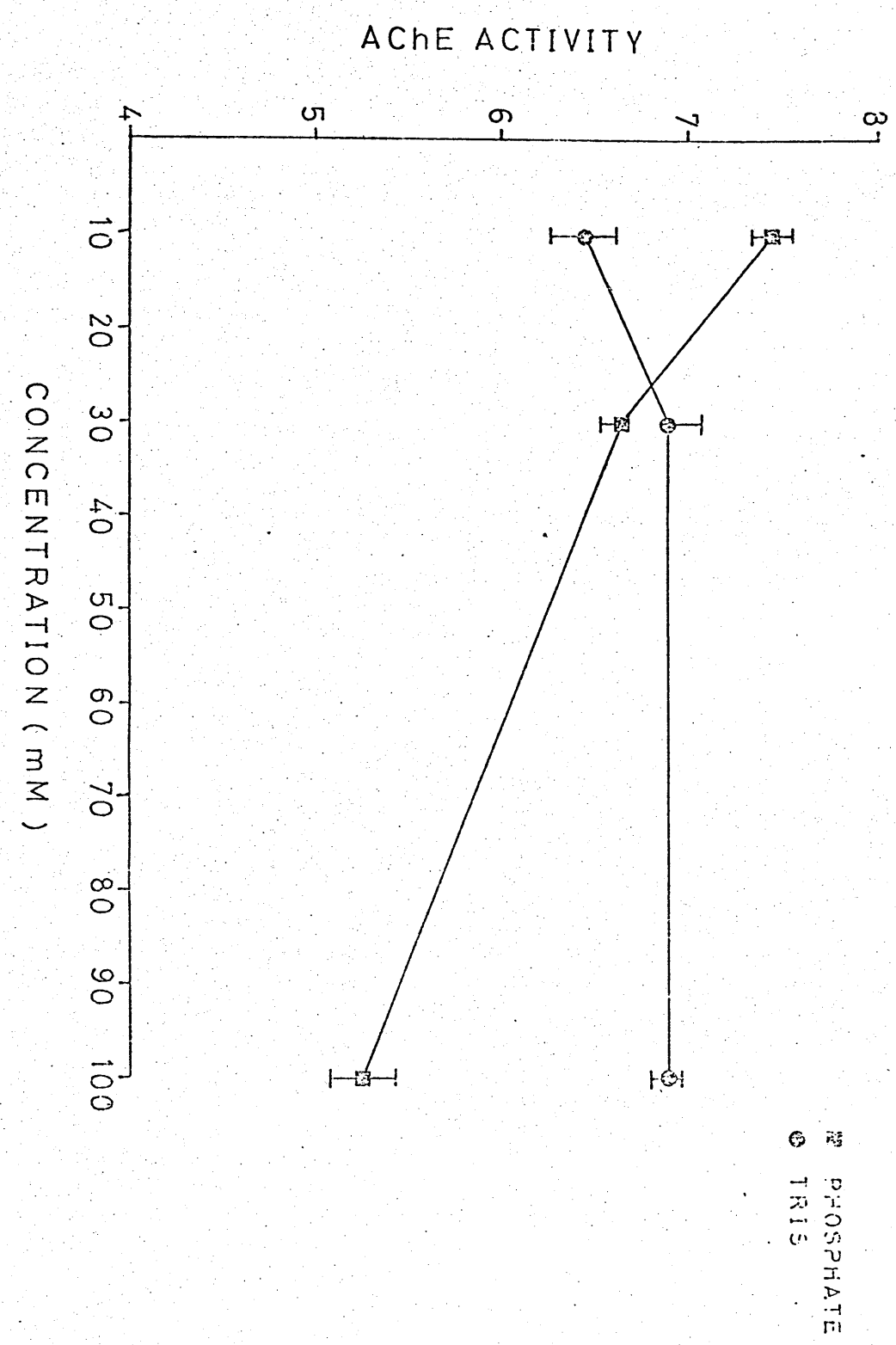

AChE ACTIVITY
4
5
6
7
8
10
20
30
40
50
60
70
80
90
100
CONCENTRATION (mM)
PHOSPHATE
TRIS

Fig. 14a

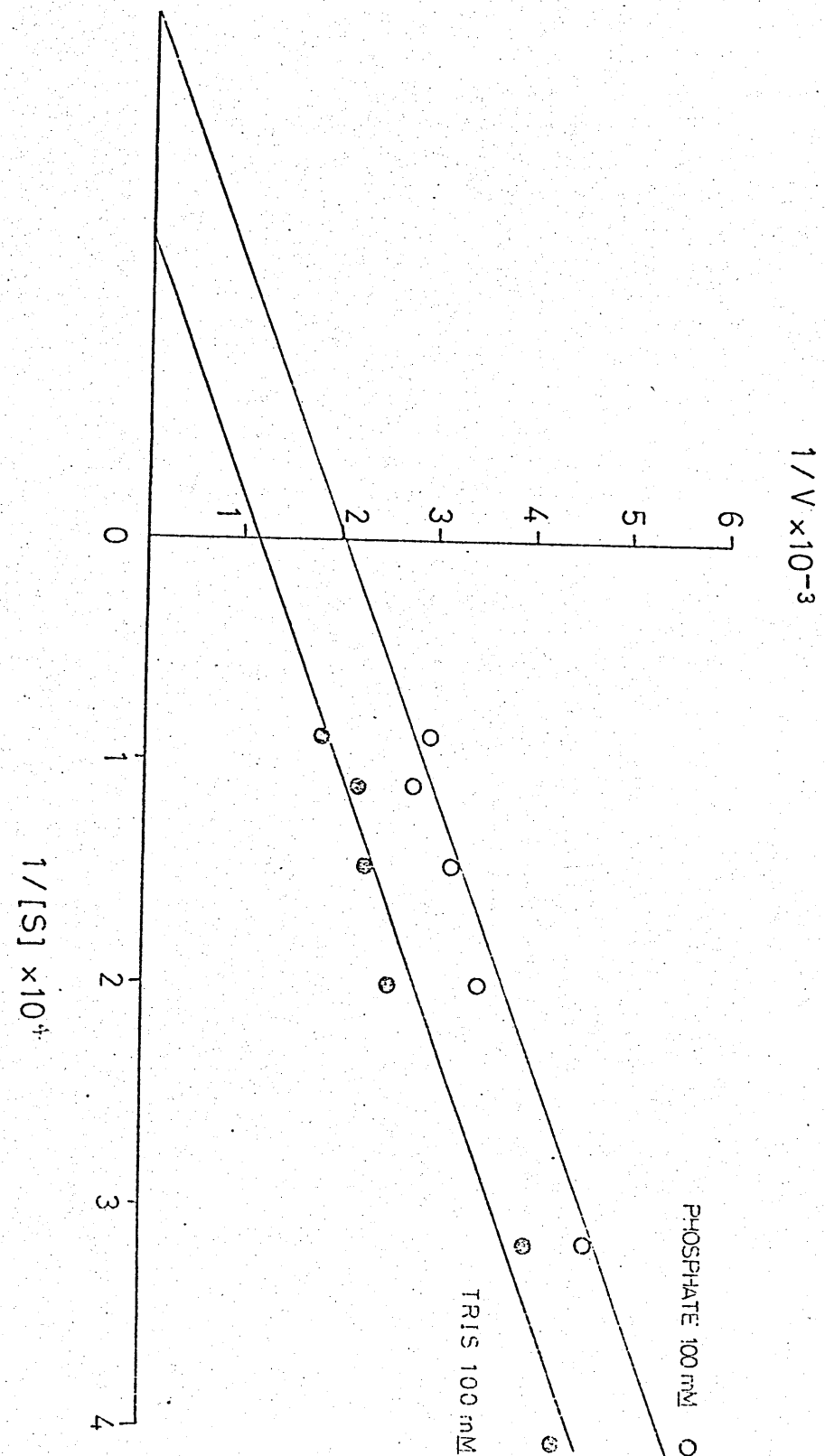


Fig. 14b

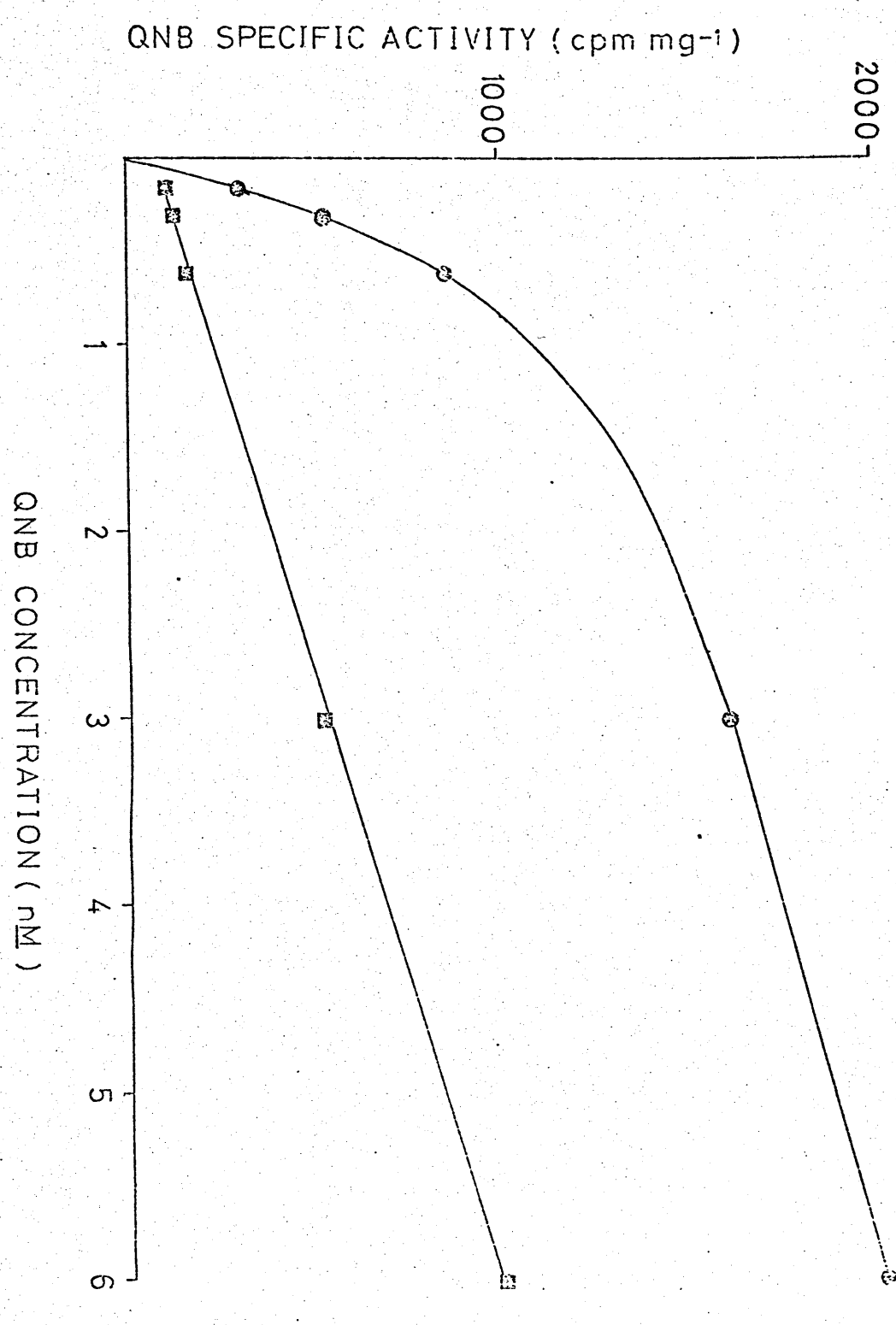

QNB SPECIFIC ACTIVITY (cpm mg-1)
1000
2000
QNB CONCENTRATION (nM)
1
2
3
4
5
6

Fig. 15

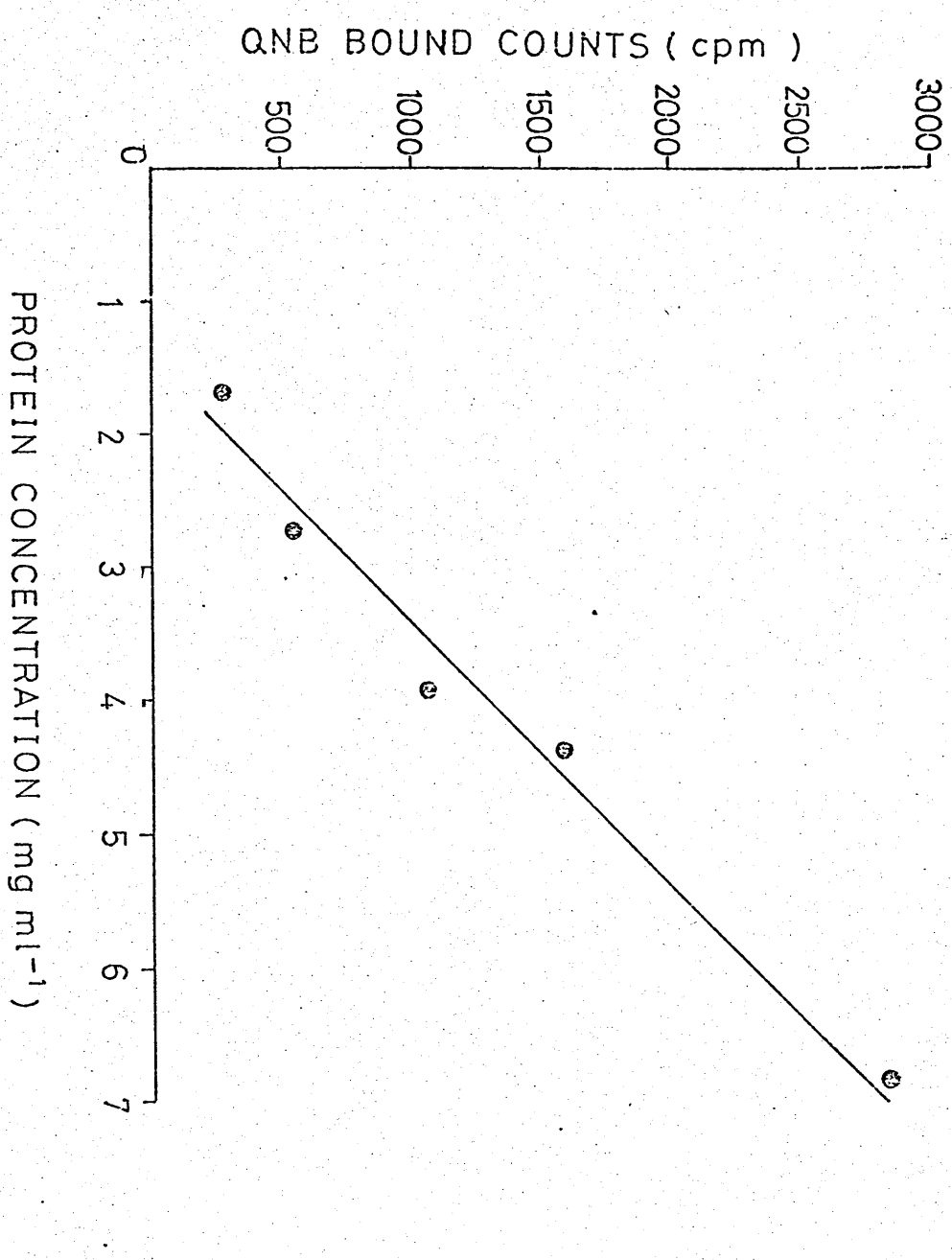

QNB BOUND COUNTS (cpm)
0
500
1000
1500
2000
2500
3000
PROTEIN CONCENTRATION (mg ml⁻¹)
1
2
3
4
5
6
7

Fig. 16

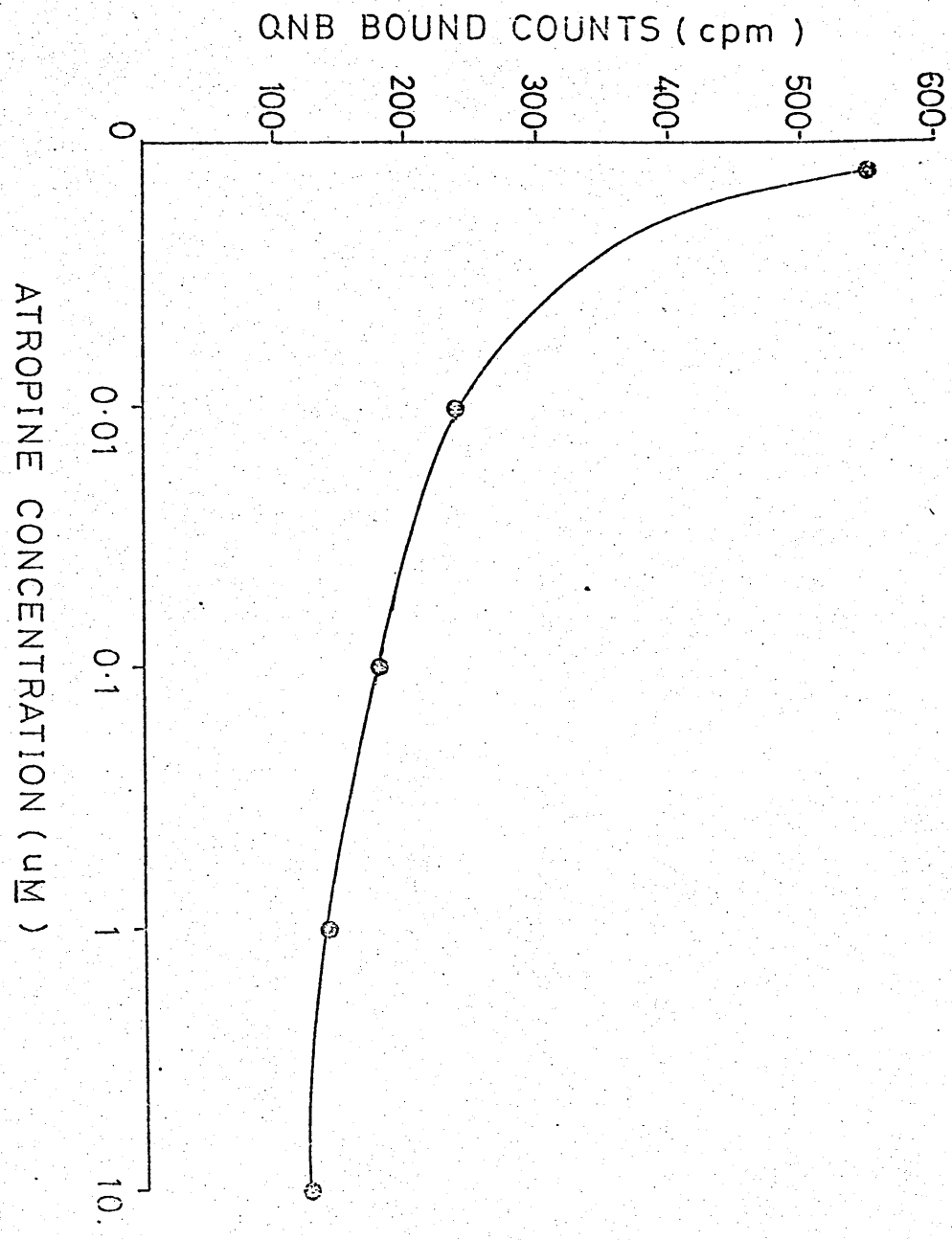

QNB BOUND COUNTS (cpm)
100
200
300
400
500
600
0
0·01
0·1
1
10.
ATROPINE CONCENTRATION (μM)

Fig. 17

LEGENDS

Fig. 2

Diagram to show T.S. section through and plan of the behavioural chambers. Measurements are in cm, and arrows refer to direction of airflow.

Fig. 3

Diagrams of red stimulus (above) and yellow stimulus. Measurements are in cm. Both stimuli are reproduced in the same scale.

Fig. 4

The 'railway'. The measurements along the track are in cm.

Fig. 5

Diagram of sagittal section through neonatal avian brain, to show dissection scheme. Traced from stereotactic atlas of pigeon brain (Karten and Hodos 1969). Key for this figure accompanies the diagram.

Fig. 6

Flow diagram of an experiment to show the metabolic fate of ^{14}C derived from radiocarbon uniformly labelled L-leucine injected pericardially into neonatal chick. For clarity wash steps are not included in this flow diagram but are described in the text.

Fig. 7

The 2.3 cm DEAE cellulose discs are described as capable of being loaded with 100 μg of protein each (Borisy 1972). Since the chick samples contained between 100-400 μg in a 100 μl aliquot, several discs, possibly 3-4, would be needed for each assay. This was checked. 100 μl aliquots of homogenate prepared from neonatal chick fore + midbrain containing 370 μg protein were incubated for 1 hour with ^{3}H-colchicine to give a final concentration in the assay mix of 2.5×10^{-6} M and then pipetted onto varying numbers of stacked, premoistened discs, washed 5 times to remove unbound radioactivity with phosphate buffer as described in the text. The discs were placed into scintillation cocktail and counted. The results were calculated as colchicine bound counts (cpm) and expressed as the means $\pm$ SEM of 3 determinations. There was no significantly greater retention of bound counts with 6 than with 4 discs, although with successively fewer discs the counts retained did decrease. On the basis of this, 4 discs were used for all subsequent experiments.

A similar experiment which used 4 discs but varied the number of washes with 5 ml aliquots of phosphate buffer from 0, 1, 2, 3, 5, to 10, indicated no difference in retained counts between 5 and 10 washes, though free radioactivity was still present at lower values. A graph of this data is not presented.

Fig. 8

The time of incubation required to ensure that the binding of colchicine with tubulin had gone to completion was found by incubating 100 μl aliquots of a homogenate containing 130 μg protein prepared from neonatal chick fore + midbrain for various periods of time with 2.5×10^{-6} M colchicine. Aliquots of the incubate were pipetted onto 4 pre-washed stacked discs, washed 5 times to remove unbound alkaloid and counted as previously described. The results were calculated as colchicine bound counts (cpm) and expressed as the means $\pm$ SEM for 3 determinations.

Although there was a slight increase in the amount of colchicine bound between 1 and 2 hours of incubation, clearly most of the binding occurred in the first hour. By adopting an incubation time of one hour very nearly maximal binding is achieved and little risk of inaccuracy would occur should the timing of the incubation be slightly protracted. Since it was comparative rather than absolute differences in binding that were of interest it was felt unnecessary to prolong the incubation period to the full two hours. In any case the binding of the colchicine tubulin complex to the DEAE discs probably only measures about 70% of that determined by similar ion exchange columns (Borisy, 1972).

Fig. 9

Originally the intention had been to measure the colchicine binding activity in material stored at -20 C which remained from the leucine incorporation experiments. When the first such assays were run however, the values obtained were much lower than those found with the pilot studies on fresh tissue, so an investigation was conducted of the effect of freeze-thawing and storage time on colchicine binding.

Homogenates were prepared from 2 fresh neonatal chick forebrains dissected in the conventional manner and colchicine binding assays were performed in the usual way after varying the number of successive freeze-thaw cycles the tissue was subjected to. Samples were thawed at room temperature (about 21 C) with

frequent agitation, and as soon as little frozen homogenate remained were placed on ice. Aliquots were removed and the samples immediately returned to the deep freeze at -20 C. The experiment was conducted over 5 successive days, with a single freeze-thaw cycle each day. The protein concentration was estimated in the fresh material, and the results calculated as colchicine binding specific radioactivities (cpm mg^{-1} protein). Each point represents the means $\pm$ SEM of 4 determinations.

Clearly after one freeze-thaw cycle colchicine binding was markedly reduced, although unthawed samples could be stored for several weeks without loss of binding (data not presented). As a consequence of these findings a separate study was undertaken to assay microtubule protein.

Fig. 10

Since the colchicine binding capacity of chick brain homogenate decays with time (Bamburg et al 1973) and the incubation time is fairly long compared with the half life reported for colchicine binding activity, in order to ascertain the initial binding capacity (IBC) it is possible to preincubate homogenates for varying lengths of time, plot the rate of decay and extrapolate back to zero time. Undertaking an estimate of IBC in this way for each of the samples generated was considered impracticable, since the experiment generated 250 samples. A minimum of 3 time points, each assayed in triplicate would thus entail 2,250 separate determinations. As a compromise a pilot study was done to determine the decay rate for neonatal chick brain under the conditions pertaining in my system, so that some estimate could be attempted of IBC if necessary. In point of fact, for the purpose of the present experiments the chief focus of interest was any differences between various conditions of birds rather than any absolute determinations. The assumption implicit in this compromise is that neither as a result of development over the first two days post-hatch, nor as a result of training does the decay rate alter.

Colchicine binding decay is a first order reaction and dependent on pH, ionic strength and the concentration of tubulin. The half-life of the complex increases with the concentration of the protein. Above 120 $\mu g\ ml^{-1}$ the increase flattens out. At the protein concentrations of the experiments typically conducted (1-4 mg ml^{-1}) the tubulin concentration would be in the order of 100-400 $\mu g\ ml^{-1}$ over which range little change in the decay rate is to be expected. Between 21-40 days

of the chick
from the start of incubation, that is, over the time span of all the experiments, there is no change in the half-life of the soluble tubulin-colchicine complex, nor the initial binding capacity (Bamburg et al 1973).

Aliquots of homogenate prepared from day old chick mid and forebrain were pre-incubated at 37 C for varying periods of time. Following this ^{3}H-colchicine in cold carrier was added to give a final alkaloid concentration of 2.5×10^{-6} M and this mixture incubated for a further hour at 37 C. Incubates were placed onto discs and washed in the usual way. The samples were assayed for protein, the results calculated as bound colchicine specific radioactivity and expressed as means $\pm$ SEM of 4 determinations, each estimated in triplicate.

The decay was found to be linear with a half-life of 195 minutes which is consistent with the values of 163 and 226 minutes for the half-life of colchicine binding decay in soluble and particulate fractions respectively, of 17 day old sonicated chick brain (Bamburg et al 1973).

Fig. 11

Flow diagram for the assay of AChE using an autoanalyser automated method. The numbers are flow rates in ml min^{-1}. For details see text.

Fig. 12

Neonatal chick forebrain was homogenized in 5 ml of 155 mM NaCl and aliquots were assayed manually for AChE activity as described earlier in the chapter, after varying the time of incubation at 37 C. Each point represents the mean of 3 determinations. The enzyme activity was expressed in arbitary units (optical density at 412 nm x 10^{-2}). No control for non-enzymic hydrolysis was done in this experiment, so while the assay is clearly linear over the 30 minute incubation period, when extrapolated back the curve does not pass through the origin but intercepts the y axis at a point which represents the contribution made non-enzymically. An incubation period of 20 minutes was decided upon which was well within the linear range for the protein concentration used.

Fig.13

To ensure that the assay was conducted with sufficient substrate present neonatal chick forebrain homogenized in 155 mM NaCl was incubated with varying final

concentrations of acetylthiocholine iodide in the incubation mixture. Assays were conducted in duplicate as described previously.

Saturation occurs at around 4×10^{-4} M with a $K_m = 9.0 \times 10^{-5}$ M. This is of the same order of magnitude as other values published for the enzyme from a number of sources, though the accuracy of such a determination from a saturation curve cannot be high (see Fig. 14 b).

Fig. 14 a and b

Early pilot studies to investigate the effect of varying the ionic strength on AChE activity by altering the phosphate buffer concentration showed that increasing the phosphate buffer concentration decreased the enzyme activity. This was not due to changes in ionic strength since varying the concentration of Tris buffer over the same range 10-100 mM was without effect (Fig. 14 a). No difference was seen between sodium phosphate and potassium phosphate buffers, ruling out a cation mediated phenomenon. The pH was kept constant at 8.0 throughout these experiments. The phenomenon could not be observed in 50 day old rat cerebral cortex. The inference that the phosphate ion is an inhibitor of neonatal chick brain AChE was studied more formally by kinetic experiments.

Aliquots of fresh chick whole forebrain homogenized in glutamate buffer at pH 6.8 were suspended in either 100 mM sodium phosphate or Tris buffers at pH 8.0. 100 μl aliquots were incubated with varying concentrations of acetylthiocholine iodide. The assay was conducted manually by the method previously described. The results are shown as a double reciprocal plot, each point is the mean of 6 determinations (Fig. 14 b). For the enzyme in Tris buffer the $K_m = 7.5 \times 10^{-5}$ M, which accords with values for AChE from a number of sources (Anand et al 1976), and is slightly higher than the 3.3×10^{-5} M quoted for 20 day old chick embryo brain acetylcholinesterase (Iqbal and Talwar 1971).

The Lineweaver-Burk plot gave uncompetitive inhibition kinetics which means that the phosphate binds to the enzyme substrate complex and not to the free enzyme. The inhibition is slight and therefore unlikely to be physiologically important.

Fig. 15

Chick forebrain was homogenized in 0.05 M Na-K phosphate buffer at pH 7.4

giving a final protein concentration of 2.36 mg ml^{-1}, and incubated with varying final concentrations of ^{3}H-QNB either in the absence or presence of 12.5 μM atropine sulphate; binding being determined as detailed previously. Both atropine displaceable and non-displaceable radioactivity is plotted, as specific radioactivities (cpm mg^{-1} protein). Each point represents the mean of 3 determinations.

The atropine displaceable radioactivity (circles) is saturable, whereas the 'non-specific' binding (squares) increases linearly with ligand concentration. Such results are in agreement with the findings of Yamamura and Snyder (1974), using rat brain homogenates. A working ligand concentration of 6 nM was adopted which although representing a somewhat higher level of 'non-specific' binding then lower concentrations did ensure that most of the available receptor sites would be saturated, and would help to eliminate any errors due to pipetting and incubation time variation.

Fig. 16

To determine the linearity of ^{3}H-QNB binding with protein concentration a chick forebrain was homogenized in 5 ml 0.05 M Na-K phosphate buffer at pH 7.4 and 5 dilutions were prepared. Binding was determined using a final ligand concentration of 6 nM, either in the absence or presence of 12.5 μM atropine sulphate as described earlier in this chapter. Protein was estimated by the method of Lowry et al (1951). A plot of atropine displaceable radioactivity against protein demonstrates linearity over the range of protein concentration used.

Fig. 17

Chick forebrain homogenate was incubated with 6 nM ^{3}H-QNB and varying concentrations of the muscarinic receptor antagonist, atropine sulphate, and ligand binding determined in the usual way.

The molar concentration of the alkaloid needed to displace half of the specifically bound counts, ED_{50} = 4 nM. This is somewhat greater than the ED_{50} values for atropine quoted for the rat, which range from 1-2 nM (Yamamura and Snyder 1974). A small proportion of binding could still be displaced by increasing the concentration of the alkaloid from 1 to 10 μM, and to ensure that all specific binding was monitored 12.5 μM atropine was utilized in all successive experiments.

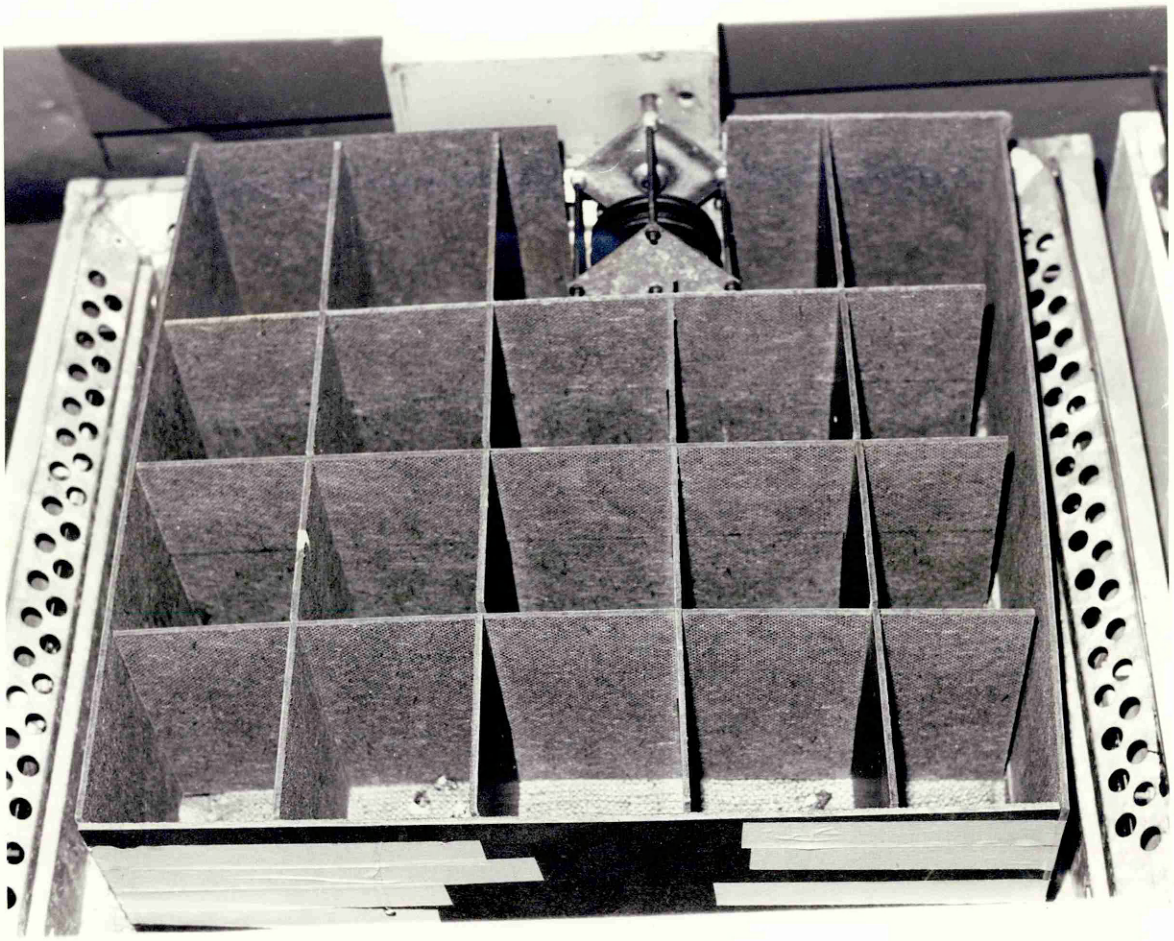


Plate 1

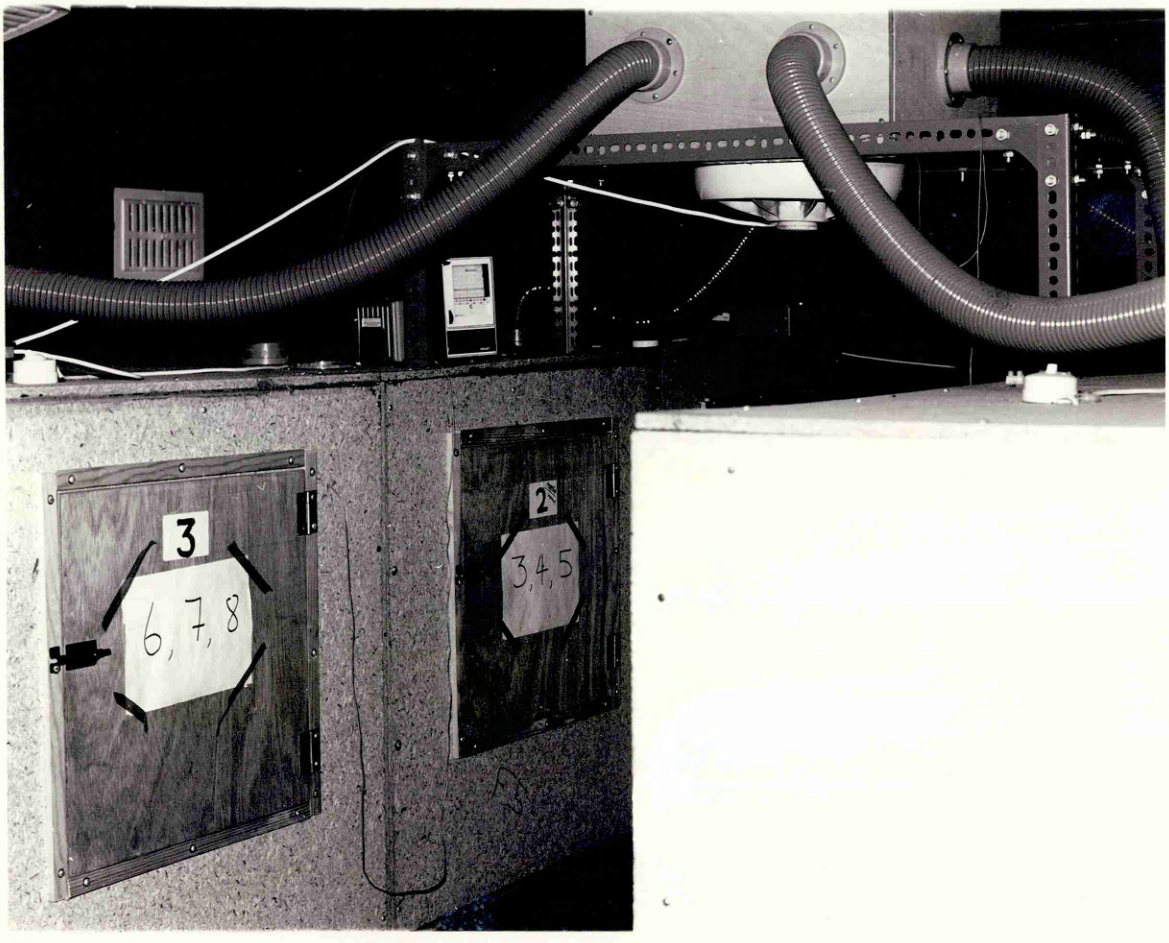
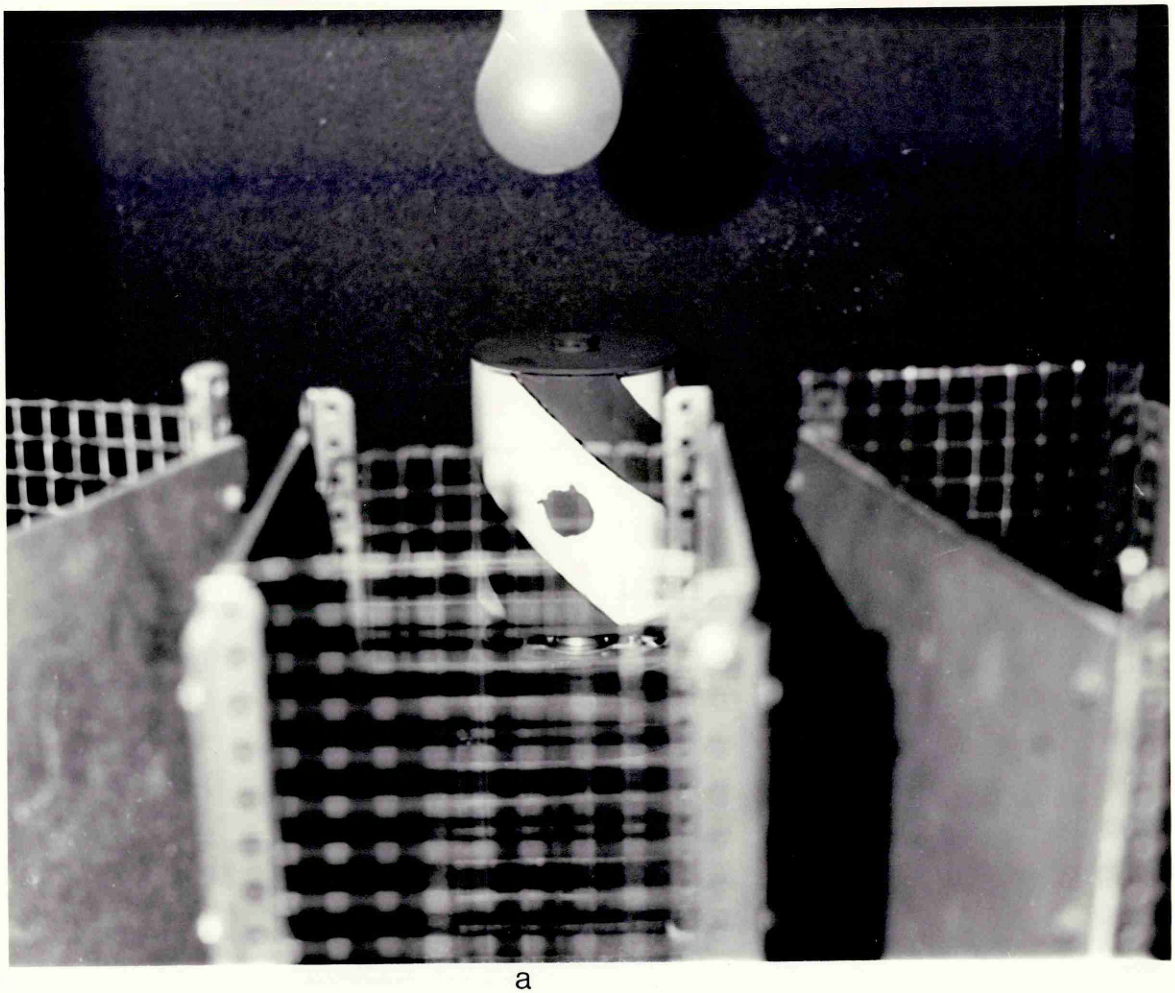
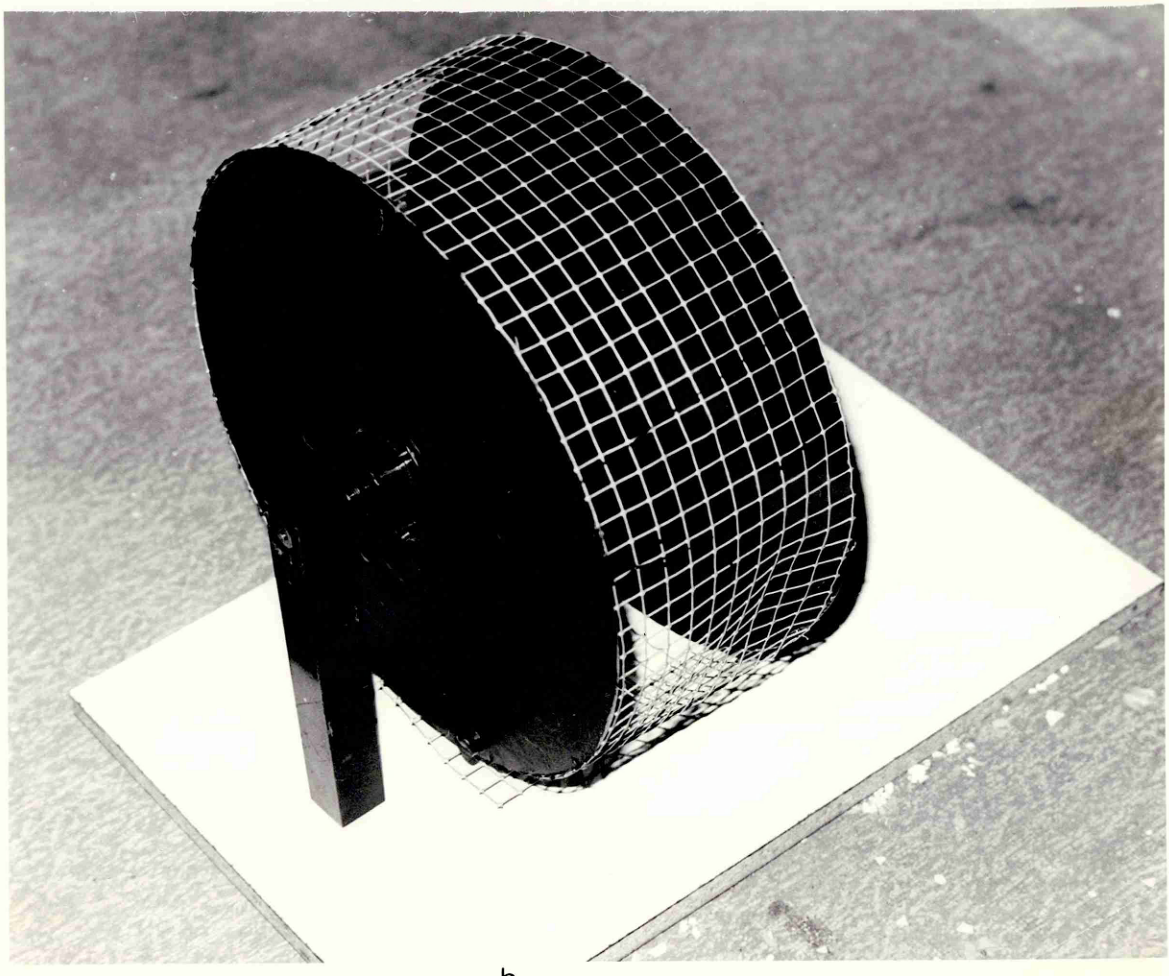


Plate 2

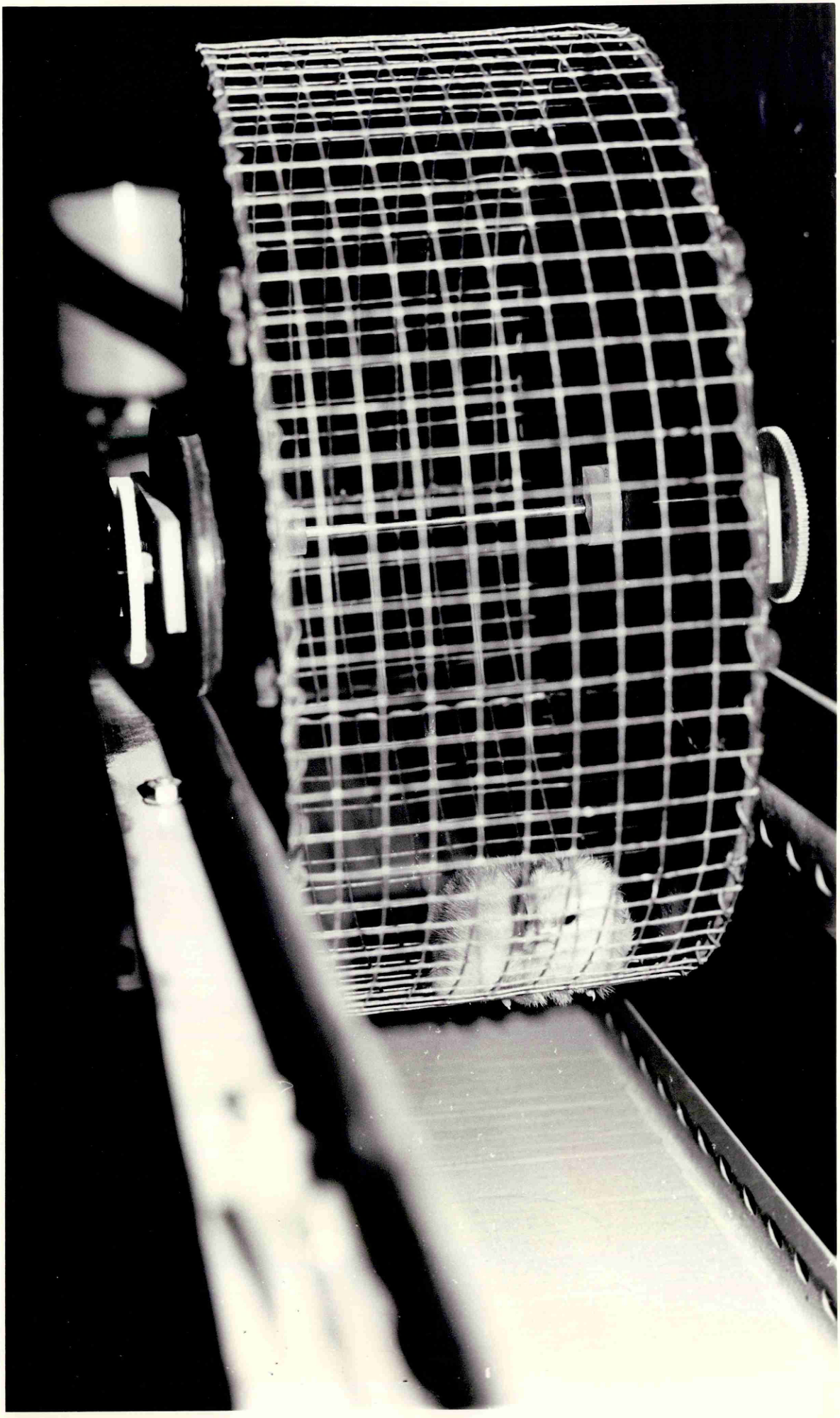


a



b

Plate 3



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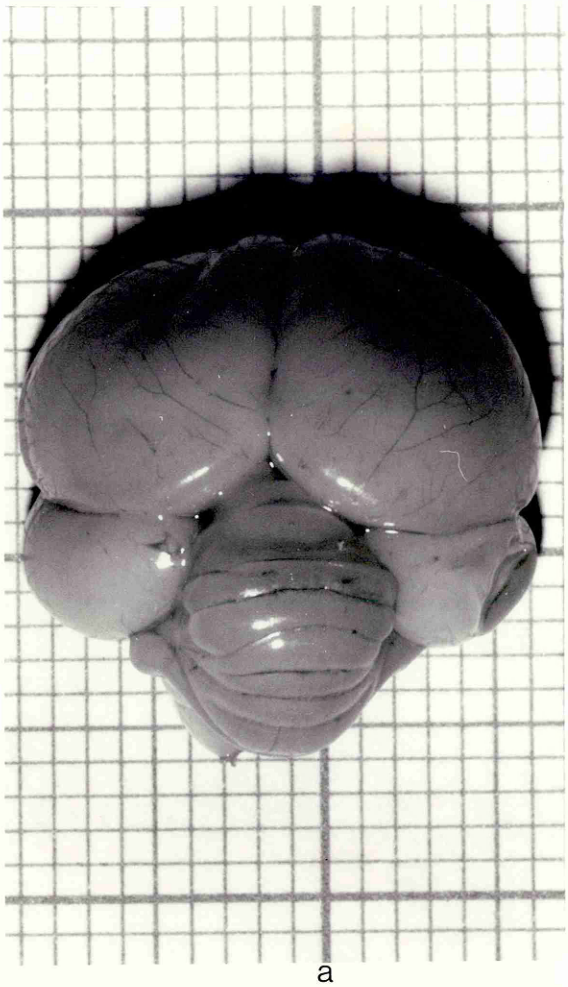

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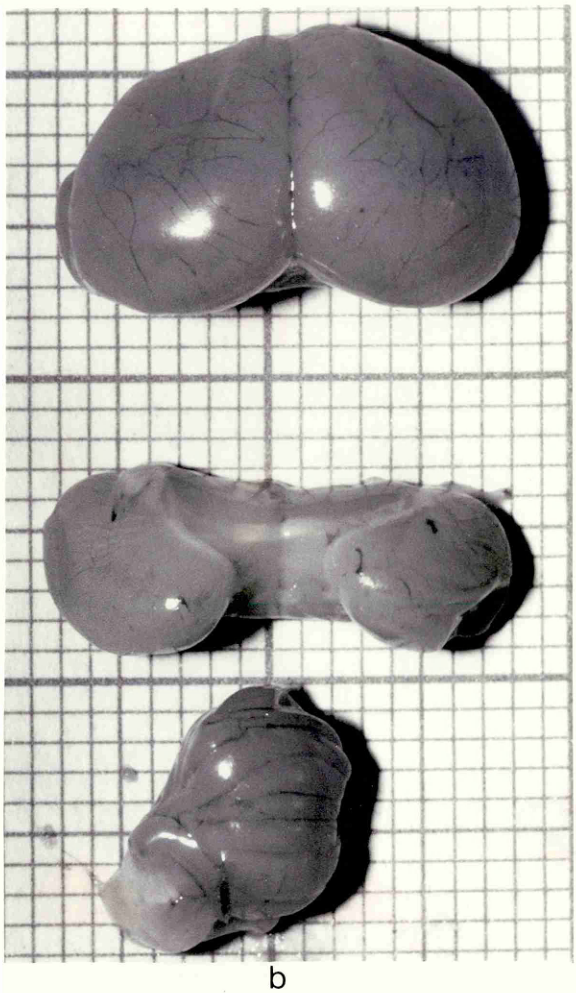
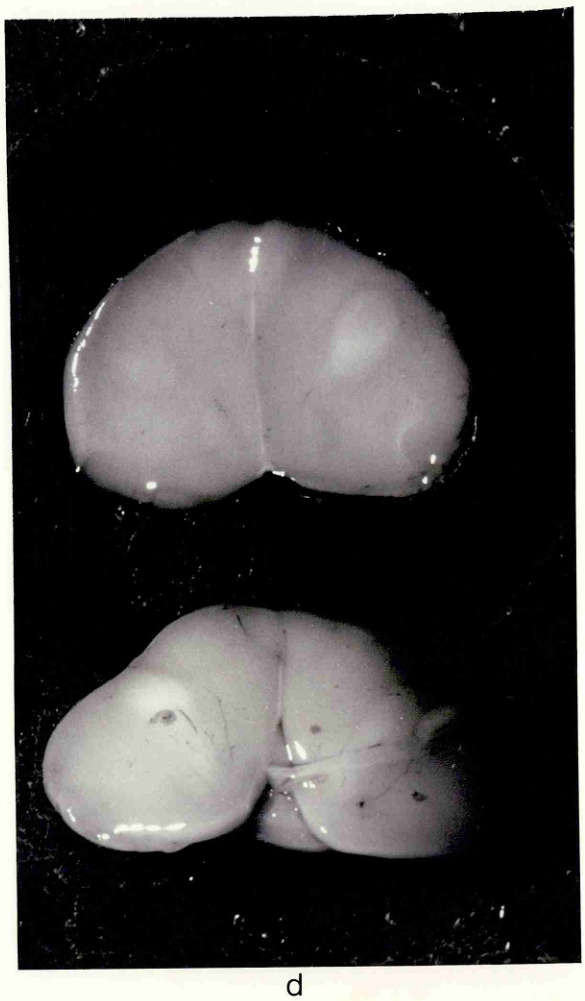


b

c



d

Plate 5

CHAPTER 5

PRECURSOR INCORPORATION INTO PROTEINS AND COLCHICINE BINDING

5.1 Lysine incorporation studies

In this first series of experiments an attempt was made to replicate the finding of an increase in ^{14}C-lysine incorporation into the anterior dorsal forebrain of chicks exposed to an imprinting stimulus (Hambley et al 1977) using newly constructed apparatus, which differed considerably from the original training equipment. Pilot behavioural studies were undertaken to investigate the efficacy of the training procedures to generate imprinting behaviour.

Chicks were hatched and collected as described in the methods section (4.1) and when 18 $\pm$ 2 hours old they were transferred to a dark brooder at 30 C where they remained for one hour, the lower temperature ensuring that they were awake and alert when training began. Training consisted of 90 minutes constant exposure to either a red or yellow stimulus placed 27 cm distant from the chick pen. Chicks were tested on the railway for a 5 minute period one hour (T1) and 12 hours (T12) after the conclusion of training. This behavioural protocol is shown in Fig. 18a The mean preference scores at T1 for both red and yellow trained animals were low (Table 2) indicating that no particular preference was shown. By T2 the yellow birds showed a small preference for the familiar, but the red birds typically showed a preference for the novel of the two stimuli. About one half of the yellow trained birds showed a preference for the familiar, but of the red trained birds, although 43% preferred the familiar stimulus when tested one hour after training, on retesting 12 hours later only one quarter indicated a familiar preference.

For the incorporation studies a methodology was adopted which closely followed that used by the original experimenters (Fig. 18b). Chicks, 18 $\pm$ 2 hours old were kept in darkness at 30 C for one hour prior to either exposure to an orange flashing light or being kept in the dark in an imprinting chamber for varying periods of time. At the conclusion of these treatments birds were injected with 5 μCi (U-^{14}C) lysine and returned to the dark brooder at 30 C. After 20 minutes they were killed by microwave irradiation, their brains were removed and dissected into four regions as described previously. For some experiments only the anterior roof was retained. Samples were homogenized with a glass-teflon homogenizer in 5 ml of 155 mM NaCl and aliquots removed for determination of acid insoluble and total radioactivity and

for estimation of total proteins. The findings are summarized in Tables 3-6.

Since no significantly increased ^{14}C-lysine incorporation had been observed in the anterior forebrain roof at one hour (Table 3), other times were explored to investigate the possibility that the timing of cerebral events had been hastened or protracted by the modification to the training procedures. Birds were either trained or kept in darkness for 30, 45 or 90 minutes. The rest of the protocol was unchanged. Only the anterior roof region was examined in each case. No differences between stimulus exposed birds and dark animals were significant at any time point (Table4), but there was a consistent trend for a reduction in incorporation with the length of time spent in the imprinting chambers for both stimulus exposed and dark birds. This trend is also evident in the data of Hambley et al (1977) between 30 and 60 minutes in all brain regions, though it was reversed by 120 minutes.

Finally a complete description was sought by studying all brain regions with 30, 60 and 90 minutes of training (Tables 5 and 6). It is noteworthy that at 60 minutes, for the anterior forebrain roof, base and midbrain, the stimulus exposed birds have approximately 20% higher ^{14}C-lysine incorporation than their dark maintained counterparts, though none of the differences reaches significance when estimated by analysis of variance.

Once again there was a reduction in incorporation with the length of time spent in the training apparatus, in all brain regions, so that the differences in incorporation between 30 minutes and 90 minutes was significant for all brain regions (Table 6). Since it occurred in exposed and dark birds alike, an effect of light stimulation is ruled out. Although exposed birds would have experienced different periods of noise generated by the stimulus, for during these early experiments the imprinting stimulus motors were turned off in the dark chamber, as dark and exposed birds both showed the effect this cannot be responsible for the differences. One possible explanation remains, that being an interaction between two successive handling experiences. Birds had two handlings which were separated by their respective training intervals; the first admitting them to the training chamber and the second removing them for injection. The final handling 20 minutes later can probably be discounted as it took place only seconds before the chicks died. It appears then that the closer together were the handlings which spanned the training period the higher was the incorporation rate of the amino acid in all brain regions, and associated changes in the acid soluble

radioactivity fractions were conspicuously absent. It is interesting in this connection that handling has been shown to increase lysine incorporation, also without any effect on free pool lysine radioactivity, in mice (Rees et al 1974).

It seemed likely that the trend towards an anatomically generalized increase in incorporation at 60 minutes might be produced by some aspect of the training rather than simply light exposure, and that this training effect was being partly masked by the fact that few birds, as shown by the low preference scores in the behavioural experiments, were learning the characteristics of the stimulus. What was needed was a way to alter the 'signal-to-noise' ratio so that training related effects, if any, could be detected. The most obvious way to achieve this was to generate a higher number of reliably imprinted chicks.

To improve the imprinting behaviour a number of changes were made:-

1. The imprinting chambers were rebuilt to give a minimum distance between the stimulus and the chick pen of 50 cm. Possibly birds confined too close to the stimulus have little motivation to either approach the stimulus or to learn its characteristics (Pat Bateson, personal communication).
2. The yellow stimuli were reduced in size from 20 x 20 x 10 cm to 18 x 18 x 7.5 cm.
3. Electronic timers were built into the stimulus control circuits so that in every 10 minutes the stimulus motor and light was off for 1 minute. This was designated 9+1 training. It was an attempt to increase the chicks' alertness during exposure by limiting habituation to the stimulus.
4. Chicks were given 30 minutes exposure to diffuse light from a 100 W tungsten bulb approximately 1 m overhead at least 30 minutes prior to the start of training. This was termed 'priming' and the rationale behind this procedure has been described previously (2.2.7).

An investigation of the efficacy of these changes on imprinting behaviour was undertaken. The protocol is shown in Fig. 19a and differs from the previous one only to the extent of incorporating a 30 minute period of 'priming' before the training. The results are tabulated in Table 7. A comparison between these findings and those from the previous behavioural experiments suggests an increase in the mean preference scores, especially for the red birds at T12, as a consequence of the equipment alterations, and which arose because a greater number of the chicks were reliably imprinted. Because no direct comparison was possible between the two apparatus designs (using the second had necessitated dismantling the original)

an alternative explanation might be that over the time it took to complete the reconstruction the chicks' behaviour, which is known to display seasonal variation, had altered.

Notwithstanding these qualifications it was of interest to see whether the improvement in the behavioural measure was reflected biochemically. A study was commenced which it was also hoped would enable a dissection of training related effects from those due to light exposure. The protocol used is shown in Fig. 19b

Chicks were randomly assigned to one of four groups. EP were exposed to an imprinting stimulus at least 30 minutes after 'priming' with a diffuse light for 30 minutes. ED were exposed to an imprinting stimulus but with no previous 'priming'. DP having first been 'primed' were maintained in the dark at the time when the first two groups were being exposed to the imprinting stimulus. DD consisted of birds which had no exposure to an imprinting stimulus and no 'priming'. Each group was further subdivided into two, with one either exposed or dark maintained for 30 minutes and the other similarly treated for 60 minutes. All groups received identical handling. The ambient temperature for the chicks was kept at 30 C throughout the experiment. Immediately after their respective treatments birds were injected with 5 μ Ci of (U-^{14}C) lysine and killed 20 minutes later by microwave irradiation. All the brain regions were dissected out as described previously and homogenized in the same manner as before. The homogenate was sampled for acid insoluble and soluble radioactivity and for protein estimation.

The results are given in Tables 8 and 9. There are no significant differences in any brain region between any of the groups at either 30 or 60 minutes. Probably the most striking feature about this data is that the level of incorporation which had occurred 20 minutes after the end of the experience, almost regardless of what it was and whether it lasted 30 or 60 minutes, varied consistently across the different brain regions; being greatest in the anterior roof > posterior roof > forebrain base > midbrain. In other words, even moving the chicks from one dark environment to another succeeded in generating different rates of incorporation in differing anatomical locations. The significant differences are documented in Table 8. The same trend occurred in the earlier experiments with the original training chambers though it was not so marked as to be significant. In none of these experiments would it seem to be an artifact of differential precursor availability in the various brain regions (Tables 6 and 9). Examination of the acid soluble radioactivities

indicates that, if anything, there is significantly less free radioactivity in the anterior dorsal forebrain than the other regions (see Table 9), yet this is the area which showed the most incorporation. Presently it is impossible to assign a reason for these anatomical variations, which are largely unaffected by visual stimulation.

5.2 Behavioural studies

Problems clearly remained and in order to be more certain of exactly how reliably birds were being imprinted, and so could be expected to show biochemical evidence of training, what was needed was an experiment in which birds could be tested following exposure to the imprinting stimulus. To determine the most effective possible design for such an experiment which might further improve imprinting behaviour, additional work was undertaken to answer certain questions.

The first (the general protocol for these experiments is illustrated in Fig. 20) simply considered the possibility that one hour was insufficient time for the birds to imprint. Two hours was selected as an alternative. Given longer the chicks might become unduly stressed as a result of the relatively cool training chambers (30 C) and earlier pilot studies had suggested that training at 33 C considerably worsened the behaviour, maybe because animals became sleepy at the higher temperature. A way to overcome this might have been to expose birds for several brief periods, interspersed with time spent in the dark at 33 C until the total exposure time equalled 2 hours. This would have necessitated much more handling which, given the previous results I was reluctant to do, and in any case such an experiment would have been practically impossible to execute since a 5 minute delay between manipulations on each chick would have been needed to ensure that they were all tested at the correct time.

The second question was directed to finding out what time after a training session it was best to test the chicks. Too soon and the animals might be too fatigued to perform well, or alternatively might be habituated to the stimulus and so be poorly motivated to exhibit approach and following responses. Left too late, and with only a short period of training some forgetting might occur, so that the birds would not be able to discriminate properly between the familiar and a novel stimulus. Another possibility could be that after spending a long time in the dark subsequent to training,

chicks might suffer from sensory deprivation. To some extent the maximum time between training and testing would need to be limited by consideration of the chicks' nutritional state, and would need to avoid confounding the experiment either by having hungry or thirsty chicks, or the requirement to feed and water them, with all the attendant sensory experience which that implies. In practise then, any experiment could not run much over about 2 days post-hatch. These questions were answered by a series of experiments with a common design, (Fig. 20).

They involved training birds on either the red or yellow stimulus for either 60 or 120 minutes and testing the animals at 3 times. The test times adopted were:- immediately after the end of training (T0), and one hour (T1), or 24 hours (T24) after training. The results of this experiment are summarized in Tables 11 and 12, where the following features are of interest. Whenever the red trained birds were tested they showed a preference for the familiar, regardless of the length of the training period. After only a single hour of training the mean preference score 24 hours later was lower than it had been immediately after training, but after a 2 hour training session the mean preference score increases as the training to testing interval becomes longer. For both training times however the scores at T24 are remarkably similar. It may be that the scores at T0 reflect transient fluctuations in performance which are related to contingencies of the training other than learning. Thus after 2 hours of training chicks might, for example, be fatigued and so perform less well in the first instance, whereas after only one hour birds are relatively undertrained and remain highly aroused when tested immediately. By complete contrast the yellow trained chicks showed a preference for novelty except when tested 24 hours after training for 2 hours, when their mean preference score would apparently indicate no particular preference. However naive birds show an inherent preference for the red stimulus, (Bateson and Jaeckel 1976), so a preference score of +1 for yellow trained birds actually suggests a definite bias towards favouring the yellow stimulus. It is documented that after a short period of exposure to an imprinting stimulus, chicks will transiently work for novelty (Jackson and Bateson 1974; see 2.5.2) and the explanation for the marked difference in response of the two groups may be that the yellow stimulus is for these chicks a less good imprinter than the red stimulus, and that this effectively protracts the whole sequence of imprinting, so that with one or two hours of training the red trained birds have passed through the transient phase of novelty preference while the yellow trained birds are actually within it (see Fig. 21). Indeed a number of factors including

previous light exposure and activity are known to alter the timing of this discontinuity in the preference curve, (Bateson and Jaeckel 1976). Obviously only a time course investigating the development of preferences for both groups could confirm the validity of this notion, but this matter was not pursued.

Another study set out to test whether birds would imprint more reliably if instead of being confined to pens during the training period they were permitted to move in free running wheels. There were two reasons for this. Although there is little evidence for a ' Law of Effort' as propounded by Hess (see 2.4) there is a correlation between the activity of birds during the first hour of a training session and preference score (Pat Bateson, personal communication), and so permitting the animals to run might generate improved preference scores. Additionally it was reasoned that transferring chicks from the stationary wire pens in which they were trained to the test wheel might involve the chicks having to adjust their posture in order to balance properly, and may also need to learn both that the wheel moves, and how to run in it. It may be of relevance that studies investigating the effects of amphetamines on motor activity of rats in wheels, have demonstrated that there were two populations of animals, 'runners' and 'non-runners'; the latter group being unaffected by the drug. More interestingly is that the 'runners' activity in the wheel improved over several test sessions because, it was suggested, they were learning to run (Iversen and Iversen 1975).

To investigate any effect of activity during training on subsequent preferences, groups of chicks were exposed for one hour to a red or a yellow stimulus while housed either in a pen or in a free moving wheel which was built to the same specifications as the wheel of the 'railway'. One pen trained bird was discarded on the basis of abnormal behaviour and gross examination of the brain revealed a remarkably small cerebellum. For those birds trained on the red stimulus, when tested one hour after the end of training (note that for this experiment no T0 was done) the mean preference score for the pen trained birds was considerably lower than the wheel trained (see Table 12) though by T24 the scores were almost identical. The findings with the pen trained animals may indicate an interaction between the two tests, i.e. the 5 minutes experience in the wheel at T1 was sufficient to ensure that their performance during T24 was as good as the wheel trained birds throughout. This provides support for the idea that the pen trained chicks have to learn to behave in the wheel. To lend weight to the argument it would have been interesting to train a

further group of chicks in pens and test them only at T24, which should have given a mean preference score much like the pen trained birds tested at T1, if the hypothesis is correct.

While both pen and wheel trained birds exposed to the yellow stimulus showed a marked preference for novelty one hour after the training, which is consistent with the previous experiment, during T24 both groups showed a preference for the familiar, the pen trained rather more so than the wheel trained. On the basis of this experiment it was decided that wheel training provided no particular advantage than pen training though I now suspect that the improvement seen in the scores of the red, pen trained birds between T1 and T24 might have been explored more fully, since definitive proof of a 'wheel learning' effect would probably have altered that decision.

Broadly speaking any subsequent experimental design appeared to be dictated by finding that to be certain of both yellow and red trained groups showing a preference for the familiar it was necessary to train the animals for two hours and test 24 hours later. While it seemed sensible to compare trained groups with dark reared chicks as controls the question arose as to whether to include birds exposed to diffuse overhead light in the training chambers as 'light controls'. An alternative, and in some ways more satisfying way of dissecting out effects which are genuinely learning related from those that are contingent on some concomitant, such as light exposure would be to use a methodology enabling a comparison of birds which had been exposed to the imprinting stimulus, yet which could discriminate only poorly between it and a novel stimlus, with those that could discriminate well. This would perhaps entail exposing some chicks to the imprinting stimulus for only a short period while others be given an additional training session.

5.3 Two day experiments: behaviour

Birds assigned to be on one of the trained groups were 'primed' with exposure to diffuse light at a minimum of 30 minutes before the onset of training. Chicks were exposed individually for 1 hour either to one of the two imprinting stimuli or housed individually in darkness. Dark birds had identical handling and treatment in all respects except the light exposure. At the conclusion of training half of the chicks in each of the three groups (determined before the start of the experiment) red trained, yellow trained and dark reared, were returned to the dark brooder where

they were retained for the next 22 hours. These were termed day 2 birds. The remaining day 1 birds were injected immediately after training with 5 μCi (U-^{14}C) L-leucine, of specific radioactivity 354 Ci mmole^{-1} (Amersham Radiochemical Centre) and kept in a dark brooder for 20 minutes, after which they were decapitated.

Day 2 birds received a further one hour of training on the second day on the same stimulus which they had been exposed to on the previous day. This was both preceded and followed by a 4 minute test on the railway. After the second test (T2) the day 2 birds were injected with ^{14}C-leucine returned to the dark brooder and killed by decapitation after 20 minutes. The brains were removed and dissected as described above and placed into coded scintillation vials on solid CO_2. Samples were stored at -20 C until assayed. The behavioural protocol is summarized in Fig. 22.

It was reasoned that T1 would provide information about how well the chicks could discriminate following a single hour of exposure to the stimulus on the previous day and which would be applicable to those birds injected and killed on day 1. Initially one hour was selected for a trial run (being subsequently retained) on the basis that after a two hour session on the first day chicks may have had little more to learn about the stimulus on day 2, with a consequent reduction of any biochemical effect which might be generated by the second days' training (cf. Bateson, Rose and Horn 1973). The training session would have been extended had any group of birds showed a preference for novelty during T1.

Now the second period of training on day 2 might be expected to improve the ability of birds to discriminate, so a comparison between undertrained 'poor' and relatively overtrained 'good' discriminators might be made. However this improvement would have to be confirmed by testing, and this test, T2, had to be immediately after the day 2 training session as the whole point of the study was to investigate the biochemistry straight after one hour of exposure to the imprinting stimulus.

Testing the birds just before the time during which they would incorporate radioisotopically labelled precursor was problematic for two reasons. Firstly because the differences in activity displayed by birds in the wheel might produce differential kinetics of precursor uptake, compartmentation and metabolism (effects of this type have been alluded too; see 3.3) which would clearly affect incorporation rates. However birds during the course of exposure in the training chambers are frequently very

active; for example by leaping at the wire mesh of the pen facing the stimulus, which seems as likely to disturb precursor kinetics as running in the test wheel. Secondly, because the test is conducted at 26 C, and both the peripheral circulatory adjustments to a rapidly fluctuating temperature and the less easily definable effects of cold stress on behaviour (e.g. 2.2.5) might result in differences in incorporation between trained and dark maintained birds. The possibility of testing birds at 30 C to offset this problem was rejected because pilot studies had shown that birds performed less well in the test at this higher temperature, seemingly because they were more sleepy, and in any event it was practically impossible to keep the test room constantly at this level. Finally it was reasoned that the design could allow groups of birds either trained or kept in the dark over the full two days of the experiment to be compared with similar groups which underwent only the first day of the experiment, so enabling an assessment of any ontogenetic changes or how these may interact with the training process.

5.4.1 Two day experiments: ^{14}C-Leucine incorporation

Samples were homogenized in glutamate buffer partly to ensure suitable conditions for retaining acidic proteins on DEAE cellulose discs and partly because of the original intention to include colchicine binding studies on these samples. Aliquots of the homogenate were removed for determination of total counts, TCA insoluble counts, acidic protein fraction incorporated radioactivity and protein estimation, all as described in chapter 4.

The mean preference scores (Table 13) shown by day 2 birds during the first test (T1) indicated that in these experiments birds trained for one hour on either one of the two stimuli on the first day had little or no preference for the familiar 22 hours later. Indeed, the mean for the red birds suggests a slight preference for novelty. However on retesting immediately following a further one hour of training, birds showed a distinct preference for the familiar of the two stimulus objects, with the yellow stimulus being much more effective in generating imprinted behaviour than the red. It would appear then that according to their performance in a well validated learning measure, this experiment produced two distinct populations of chicks; the yellow trained animals being 'good learners' and the red trained 'poor learners'. Since both had received similar visual experience any comparisons between these groups would be likely to reveal any differences due to some aspect of the learning which is contingent on exposure to the imprinting stimulus. Comparisons between either of the trained groups and the dark animals might be expected to indicate

alterations which occur simply as a result of exposure to light. Furthermore, since dark reared birds were maintained on both day 1 and day 2 it was possible to dissect developmental modifications taking place over the first two days post-hatch from the effects engendered by training.

Incorporation of the radiolabelled leucine into the acid insoluble fraction, which may be taken as a measure of the total protein synthesis during the 20 minutes following treatment, gave no differences between dark birds on day 1 and day 2 in any brain region (Table 14).

As regards the acid soluble fraction, which represents unbound radioactivity, a large increase was detected in day 2 dark chicks as compared with day 1, in both the anterior forebrain roof and the midbrain. In the posterior forebrain roof and in the base, although not significant, the same trend for an increase in free radioactivity was observed. These developmental changes were reflected in the trained groups of birds. Incorporation into the acid soluble fraction was markedly increased in all regions for the red birds on day 2 as compared with their day 1 counterparts. A similar trend was seen in the yellow trained birds which reached significance in the forebrain base and the midbrain. These changes are summarized in Table 15.

For the disc retained acidic protein fraction the only developmental change in the incorporation of ^{14}C-leucine was seen in the anterior forebrain roof, which showed a substantial decrease over the first two days post-hatch (Table 16).

No significant differences in incorporation occurred between trained and dark birds on day 1 into either acid insoluble, acid soluble or acidic protein fractions.

On day 2 a 25% and a 33% increase in the incorporation of ^{14}C-leucine occurred into the acid insoluble fractions of the anterior forebrain roof and midbrain respectively, of yellow trained birds compared with dark reared animals; but neither of these is significant with one way analysis of variance (Table 14).

No changes in the amount of free radioactivity in the acid soluble fraction were detected in either of the day 2 trained groups by comparison with the dark reared controls, neither did the trained groups differ significantly from each other in this respect (Table 15).

In the anterior forebrain roof of the yellow trained chicks the disc retained acidic proteins had significantly higher incorporation of ^{14}C-leucine than either red trained or dark birds as estimated by one way analysis of variance (Table 16). There was a similar trend in the posterior forebrain roof, with a significant difference between the two trained groups, the yellow being elevated above the red, but not between the yellow trained and dark birds. When the preference scores for individual yellow trained chicks were plotted against incorporation into the acidic protein fraction a weak positive correlation (indicated by Spearman Rank Correlation Test) was evident between the measure of learning and the incorporation into the anterior forebrain roof, but in no other region (Figs. 23,24,25,26). The assumption inherent in this treatment of the results is that the relationship between the performance measure and incorporation rate is linear, and there is no a priori reason for expecting this to be the case.

One reason for the difference between the two trained groups might be that birds respond differently to red and yellow wavelengths. Behavioural evidence does suggest that the responsiveness of naive chicks is greatest to red (Bateson and Jaeckel 1974), certainly on the second post-hatch day (Taylor, Sluckin and Hewitt 1969). From a biochemical standpoint this may not be as extraordinary as it sounds. When young Rhesus monkeys were exposed to a flickering light the shorter wavelengths of light generated a greater increase in incorporation of intracisternally administered ^{3}H-lysine into proteins of cortical areas 17, 18 and 19 than longer wavelengths (Singh and Talwar 1967). Though in the present experiments the effects are in the opposite direction to those in the primate there is no a priori reason why such a differential responsiveness to the colour of light should not be responsible for any distinction between red and yellow trained birds. That it is unlikely in this case is shown by the lack of a difference between the trained groups on day one, and the existence of a correlation between the biochemical measure and the preference score.

5.4.2 Two day experiments: Colchicine binding studies

It was thought possible that the incorporation of ^{14}C-leucine into the acidic protein fraction might represent, at least in part, synthesis of microtubule protein and using colchicine binding assays to estimate the total tubulin content of homogenates might reveal changes in net synthesis. Technical problems (see Legend to Fig. 9), necessitated a further series of experiments. Four separate hatches were used for these colchicine binding studies and the behavioural methodology was altered slightly.

The first test (T1) was omitted and the assumption made that like the previous hatches, these birds would not show a significant preference for the familiar after a single one hour training session on day 1. None of the birds were injected with radiolabelled leucine and it was assumed that these birds would not constitute a distinct population to previous hatches in respect of their biochemistry. In all other matters the training schedule was identical to that previously formulated and shown in Fig. 22.

For these birds the mean preference scores for both trained groups at T2 were high (Table 17), indicating that, for these animals both stimuli were equally effective in eliciting imprinting behaviour.

On both days birds were decapitated 20 minutes after the end of training, their brains removed, dissected and placed into coded scintillation vials on solid CO_2. Samples were stored at -20 C until assayed. The samples were homogenized in glutamate buffer since the optimal stability of colchicine binding is given by the presence of sodium and glutamate ions, high ionic strength and pH 6.8 (Wilson 1970). Aliquots were removed for determination of tubulin content.

The biochemical findings may be summarized:-
Ontogenetic changes were found for colchicine binding. This decreased over the first two post-hatch days in the posterior forebrain roof and the midbrain of the dark birds. Such alterations were also seen between day 1 and day 2 for the trained groups. Decreases in colchicine binding of a similar order of magnitude were found in the forebrain base of both groups and further, yellow trained chicks had lower binding in the posterior roof on the second day. These results are summarized in Table 18 where it will be seen that similar trends exist for nearly all regions examined in all groups.

On day 2, yellow trained, red trained and dark chicks differ significantly from each other in respect of colchicine binding in the posterior forebrain roof (as estimated by one way analysis of variance). Indeed red trained birds have a significant 18% increase in binding by comparison with dark birds, and a similar trend exists between yellow trained and dark birds for the same region (legend to Table 18).

5.5 Two day experiments: discussion

The design of the two day experiments permitted a comparison between day 1 and day 2 dark birds and would be expected to reveal any developmental changes over the first two days post-hatch. Certain ontogenetic effects were indeed found.

In one brain region, the anterior dorsal forebrain, the incorporation of ^{14}C-leucine into the acidic protein fraction fell over the first two post-hatch days. This is not likely to be related to pool size since the dark chicks had higher levels of unbound radioactivity in all brain regions on day 2 than day 1. Over the same period the total quantity of tubulin as measured by colchicine binding tended to decrease. This observation is compatible with the report of Bamburg, Shooter and Wilson (1973). The decrease in total quantity of tubulin could represent increased degradation either alone or coupled with a decrease in synthesis.

With the training protocol of these experiments, a reliable preference for the familiar was only shown after the second (Day 2) training session. The protocol differs from that used earlier (e.g. Hambley et al 1977) and in addition batch variations between groups of birds over time must be borne in mind (e.g. Bateson, Horn and Rose 1975). On Day 1, trained animals showed no differences in ^{14}C-leucine incorporation as compared with those kept in the dark. The implication is that a one hour exposure to the imprinting stimulus, which does not result in development of a preference for the familiar object also has no influence on the rate of cerebral protein synthesis over the succeeding 20 minutes.

After the second training session the two groups of trained birds, although they may be regarded as having had similar visual experience, responded differently, in that the yellow trained birds showed higher preference scores than the red trained ones. Moreover it was the yellow trained birds which showed the elevated incorporation of amino acid into the anterior roof acidic proteins, when compared to red trained or dark birds. The correlation between the preference for the familiar (a measure of how well the birds learn) and incorporation of ^{14}C-leucine into acidic proteins of the anterior dorsal forebrain supports the contention that such incorporation changes may be learning dependent.

Such a finding is not without precedent. A previous experiment in which a variety of behavioural measures were made of the birds, has shown a positive correlation between the preference score and the degree of incorporation of uracil into RNA in the anterior roof (Bateson, Horn and Rose 1975; see 3.3) but in no other region. Furthermore no other behavioural measures (e.g. of latency, activity or stress) correlated significantly with the incorporation into the anterior roof.

One possible explanation for the elevated incorporation in the anterior roof of yellow trained birds on the second day might be a greater precursor availability, and as we have seen day 2 birds do show greater incorporation into the acid soluble fraction than do day 1 birds. This is an unlikely explanation, however, since similar changes in the acid soluble fraction occurred in the other brain regions, and in dark and trained birds alike. These striking modifications in free radioactivity over the first two post-hatch days are in agreement with a study which showed increases in brain pools of several amino acids, including leucine (Levi and Morisi 1971).

This work found that over the period of hatching there was a generalized increase in pool sizes of most amino acids in the brain. Notably these pools remained expanded in the adult in the cases of glutamate, glutamine, aspartate, and GABA, and was taken to indicate maturation of enzyme and high affinity uptake sites for these substances. In heart and liver such pool changes were restricted to a few amino acids e.g. glycine, valine, taurine and leucine. Certain other pools were reduced in these tissues.

It was proposed that the brain pool alterations are a response to the lack of oxidizable substrates which occurs at the time of hatching. Early on they reflect to a limited extent plasma amino acid levels, which is not surprising since the blood-brain barrier is relatively poorly established in the neonatal chick (Roberts and Kuriyama 1968) and the later independence of plasma and cerebral amino acids was taken as evidence for its maturation.

Whilst a direct comparison of unbound acid soluble specific radioactivity and leucine pool size cannot be derived from these results, since the specific radioactivity of the pool could have altered, the close correspondence between the present findings and those of Levi and Morisi, who measured amino acid concentration, suggests that the acid soluble fractions do reflect pool sizes, at least in the dark birds.

There is tentative evidence that the availability of leucine for protein synthesis may be altered by one training paradigm. Between 2-8 hours after a swimming task was acquired by goldfish the acceptor activity of $tRNA^{Leu}$ was increased in the brain but not the liver. The effect had gone by 24 hours post-training and was not seen in 9 other tRNA species. Because leucine pool size did not change the increase was not

due to greater amino acid availability, although it could have been a result of increased amino acid turnover. All of this suggests a highly specific process.

Since the average half life for tRNA is 5 days in mammals and possibly longer in poikilotherms, it would seem that post-transcriptional modification of the molecule generates the altered activity, though what sort of modification is not known (Kaplan and Sirlin 1975).

Interestingly, in this paradigm increased leucine incorporation occurs into a cytosol fraction 2-8 hours after training, which is reminiscent, albeit with a longer time course, of the chick imprinting findings.

The tRNA population of highly specialized cells often reflects the amino acid composition of the predominant protein, and may serve as a point of translational control of protein synthesis; it can be the rate limiting step. Possibly then leucine availability might be rate limiting for the synthesis of particular proteins, and this availability is in turn regulated by the aminoacylation properties of $tRNA^{Leu}$, which are altered in some, as yet unknown way, by training.

A number of authors have reported increased incorporation of amino acids following a wide variety of learning paradigms. After a brightness discrimination learned by footshocked rats, ^{3}H-leucine was injected intraventricularly. The relative specific radioactivity (RSA) was increased in trained animals compared with active and passive controls for several electrophoretically separated soluble acidic proteins (Popov et al 1975), however densitometry of the amino black stained gels showed no differences between any groups in the electrophoretic pattern. With a different methodology, autoradiography, but the same strain of rat and training schedule Pohle and Matthies (1974) found that intraperitoneally injected ^{3}H-leucine was incorporated to a greater extent in hippocampal neurones (especially CA3 and CA4 cells) visual and cingulate cortex when compared with active controls which the authors reasoned probably suffered greater stress. However, altered precursor incorporation kinetics is by no means restricted to situations in which animals learn, neither is incorporation of precursor into proteins always elevated by training procedures. Rees et al (1974) exposed mice to one of several conditions, a training schedule, handling, buzzers, lights or footshock and measured ^{3}H-lysine incorporation into protein in brain and liver. Corrections were made for the exchange of

tritium with water. When they expressed their results as RSA's it seemed that the response of the brain and the liver to the various treatments was similar, if much larger in the latter. When the incorporation into TCA insoluble and soluble material was considered independently, however, several striking differences emerged. While none of the manipulations affected amino acid pools in liver, all except handling or a small number of footshocks produced elevations in free radioactivity with respect to quiet controls. It was found that by day 4, when trained mice had reached an asymptotic level of performance, there was a marked reduction in the incorporation of precursor into protein. Similar attenuation of response also occurred with prior experience of the buzzer in both brain and liver; the latter of which was taken as implicating a hormonal influence. The authors concluded that novelty was responsible for the biochemical effects.

A one trial passive avoidance task using the same strain of chick as that in the imprinting experiments Mileusnic, Rose and Tillson (1980) showed that ^{14}C-leucine incorporation into the anterior dorsal forebrain TCA precipitable material was increased in both soluble and particulate fractions at 30 minutes after training. Moreover ^{14}C-leucine incorporation was also shown to be elevated in soluble fraction proteins retained on DEAE cellulose discs at 0.5 and 24 hours, in the anterior roof. These authors eluted the protein retained by the discs with SDS and using PAGE found 5 bands, two of which corresponded to α and β tubulin subunits and comprised 70% of the total protein. It is important to point out that the discs were washed with a buffer of relatively high ionic strength (67 mM sodium phosphate containing 100 mM KCl) which almost certainly resulted in the loss of proteins retained on the discs under the conditions of the imprinting study.

It is a little difficult to assess the one training related increase in colchicine binding, that occurring in the posterior roof of red trained birds. The same trend was noted in the yellow trained group but it failed to reach significance. That there was no increase in the anterior roof as a result of training shows that there is no net synthesis of tubulin dimer in this region, and the increased ^{14}C-leucine incorporation must then represent an elevated turnover of tubulin or increased synthesis of other proteins. This has to be viewed in the light of the study described above which found not only an increase of ^{14}C-leucine incorporation into the anterior forebrain roof 30 minutes after training, but coincidentally an increase in the total quantity of soluble and particulate tubulin as estimated by colchicine

binding (Mileusnic et al 1980). This work does not necessarily contradict the imprinting findings, but indicates that there may be differences in the anatomical substrates for the two types of learning and also in the timing of the biochemical events, for example.

A possible role for tubulin in learning might be that microtubules are assembled to facilitate the fast axonal transport of materials from the perikaryon and so promote the growth of neurites. Cronly-Dillon and Perry (1976) cite as evidence for this view the finding that agents which promote the formation or stabilization of microtubules (e.g. D_2O) both accelerate the growth of neuronal processes in vitro. (Murray and Menitez, 1968) and facilitate learning in goldfish (Lehr, Wenzel and Werner 1970).

It is necessary to appraise exactly what changes in colchicine binding means in functional terms. Colchicine is thought to bind on a molar basis with tubulin dimer (Weisenberg et al 1968), but not to the 36S ring structures which are the precursors of microtubules, or to the microtubules themselves (Weingarten et al 1974). With the homogenization conditions employed it is likely that microtubules are depolymerized so that all the microtubule protein is present as tubulin dimer. It seems unparsimonious that plastic changes at the cellular level are brought about by the de novo synthesis of large quantities of tubulin, when potentially there are a number of mechanisms which could alter the state of tubulin and so presumably its function. For instance, Morgan and Seeds (1975) studying neuroblastoma differentiation found that in these cells a vast proliferation of neurotubules accompanies neurite outgrowth, but the amount of tubulin present in the cells remained constant throughout as determined by colchicine binding. This suggested that the differentiation was brought about by post-translational regulation of microtubule polymerization.

If it is the state of polymerization which is the important functional determinant in learning then this might be brought about in any number of a multitude of different ways.

Certain proteins which seem to be associated with microtubules may play a role in the polymerization process. One, with an electrophoretic mobility identical to a sperm tail dynein, is unlike other dyneins in not having Mg^{2+} or Ca^{2+} dependent ATPase activity (Burns and Pollard 1974). This protein has a Stoke's radius which indicates that it is highly filamentous and other workers have reported that it is

associated with tubulin through several cycles of reassembly and may contribute to the side arms which can be seen projecting from microtubules by electron microscopy (Gaskin et al 1974). While the existence of 'side arms' invokes notions of the sliding filament model of muscle contraction, which may explain a role for microtubules in mitotic spindle function or flagella motility, it is more difficult to imagine how these structures would effect axonal transport or movement of neurotransmitter vesicles to the synapse in the absence of ATPase activity. The lack of ATPase activity in brain dynein may however merely reflect lability under suboptimal preparation conditions.

A second microtubule associated protein called tau is incorporated stoichiometrically into microtubules so is unlikely to function catalytically (Weingarten et al 1975) and is thought to play a part not only in the initiation of microtubule growth but also in its continued elongation. When added to purified dimer this protein permits the polymerization of this 6S form to a 36S ring structure which then assemble into microtubules via protofilaments at 37 C but not at O C (Kirschner et al 1975).

Tubulin can undergo a number of post-translational modifications. A cAMP dependent protein kinase may be responsible for the phosphorylation of either the β subunit (Eipper 1974) or both subunits (Lagnado, Tan and Reddington 1975). Whether this enzyme activity is intrinsic to tubulin itself (Soifer et al 1975) or independent of the microtubule protein (Eipper 1974) is a contentious issue, but the finding by Lagnado and Kirazov (1975) that there is a much higher incorporation of ^{32}P into serine residues of the 36S form than the 6S dimer suggests that the phosphorylation may play a role in the transition between these states and so may regulate polymerization. Highly purified microtubules from chick brain contain a cAMP dependent protein kinase which phosphorylates a microtubule component (Sloboda et al 1975). Each 6S dimer has one tightly bound GTP and one mole which will readily exchange with free GTP in the medium. It seems possible that transphosphorylation occurs between GTP in the exchangeable and non-exchangeable site and that dephosphorylation accompanies assembly (Berry and Shelanski 1972) though the significance of this is far from clear, especially since another study found that with polymerization dephosphorylation of GTP occurs at both sites. The nucleoside triphosphatase activity does not appear to be associated with the non-exchangeably site since hydrolysis here is too slow to account for the observed GTPase activity. Indeed Jacobs (1975) concluded that the triphosphatase activity must reside not with the tubulin but with a copurifying contaminant.

Since high Ca^{2+} levels seems to inhibit polymerization this ion has been postulated to have a regulatory role in microtubule polymerization. Two workers have documented calcium binding to the 6S but not to the polymeric forms (Hayashi and Matsumura 1975), although since the physiological levels are 1000 times lower than those needed to exert an inhibitory effect it is doubtful that this is important in vivo (Olmsted and Borisy 1975).

Blocking 2 out of the total of 7 free sulphhydryl groups in the tubulin dimer abolishes the ability to polymerize (Kuryama and Sakai 1974) and oxidation of these -SH groups or their protection from oxidation by e.g. endogenous levels of glutathione, may regulate the degree of tubulin assembly in vivo. The problem currently is that these processes, some of which could conceivably provide a link between neuronal activity and the state of microtubule polymerization although extensively studied in vitro have yet to be shown to be important in vivo.

Notwithstanding all this, the half life of the soluble tubulin-colchicine complex of chicks alters with age, which Bamburg, Shooter and Wilson (1973) took to interpret as a change in microtubule protein itself. It was proposed that early in development the tubulin functioned as a mitotic spindle component during the period of rapid cell division and later it subserves a role in axoplasmic transport at a time when synaptogenesis is occurring.

Further, fetal and neonatal mouse brain will more readily polymerize than the adult form. This work (Koehn and Olsen 1978) assessed the quantity of tubulin that was assembled by the difference between the colchicine binding of a soluble fraction prepared at 0 C (favouring depolymerization) and 23 C (favouring stability of pre-existance microtubules). Two possibilities arose. Firstly that the excess tubulin present in the fetus and neonate compared with the adult represented a different species which more readily polymerizes (cf. Bamburg, Shooter and Wilson 1973) or alternatively that some factor present in neonatal brain stimulates polymerization.

Kirschner et al (1975) also has evidence for heterogeneity of tubulin. An agarose column fractionation of Ca^{2+} depolymerized microtubules prepared from day old chick brain by three cycles of reassembly was run. The majority of the protein eluted in the void volume of the column, contained both 6S and 36S forms (Y tubulin) and tubulin eluted thereafter was only present as 6S type (X tubulin). Y tubulin could

not be induced to assemble. The properties of these two species can be summarized:-

X	Y
Will not form 36S rings	will form 36S rings
High affinity for colchicine	low affinity for colchicine
Does not undergo phosphorylation	can be phosphorylated

The implications of this are that the 6S and 36S forms do not represent a simple monomer-polymer equilibrium; neither of the fractions when left regained the proportions of the two forms that had been present in the original mixture. If these results reflect the in vivo situation and are not just an artifact of the experimental methodology then a further complexity is added to the interpretation of any colchicine binding study.

Quite apart from changes in polymerization which may be complicated by the existence of two differently polymerisable species of tubulin, functional interpretations are further complicated by the compartmentation of tubulin. It exists both as a soluble cytoplasmic protein, and in an insoluble state bound to membranes. In the chick there would appear to be no differences in any parameter of colchicine binding between these two compartments, though recently other differences have been reported. The α-tubulin can be tyrosylated at its C-terminal glutamate through the action of a ligase. While this does not alter the ability of the tubulin to polymerize, and while both soluble and particulate tubulin can be tyrosylated in vitro it seems that in vivo it is only the soluble form that is tyrosylated. This suggests that tyrosylation may regulate the compartmentation of the protein between its soluble and particulate forms (Raybin and Flavin 1977; Nath and Flavin 1978). In chick brain tyrosyltubulin ligase drops markedly from day 18 of incubation to the fourth post-hatch day (Deanin, Thomson and Gordon 1976), which suggests that incorporation of tubulin into membranes may be curtailed over this time. This might mean that during the sensitive period a larger pool of soluble dimer is avilable from which microtubules are assembled, thereby increasing the potential of the neurone to transport materials from its perikaryon to the neurites. However it is worth bearing in mind that the tyrosylation is reversible, so the rate of turnover of the C-terminal tyrosyl residue may be more important than the proportion of tubulin tyrosylated at any time. Interestingly soluble tubulin is periodic acid schiff (PAS) negative and particulate

is PAS positive, reflecting non-carbohydrate and carbohydrate containing tubulins, which led Nath and Flavin to postulate that the membrane bound tubulin with a glycosyl moiety on the N- terminal end projecting from the membrane surface might function as a recognition group, and so mediate intercellular communication via reversible tyrosylation at the C-terminus located inside the cell.

The existence of particulate tubulin has been established beyond doubt. Post-junctional lattices (PJL) isolated by deoxycholate digestion of a synaptic plasma membrane fraction has been shown to have a major component which co-migrates with authentic tubulin on SDS-PAGE and which gives trypsin digested 'fingerprints' which are virtually identical to tubulin (Walters and Matus 1975). Moreover an anti-tubulin immunoperoxidase method succeeded in staining both microtubules and post-junctional lattice in rat cerebral cortex, (Matus, Walters and Mughal 1975). Microtubules have been shown to extend to within 40 nm of the pre-synaptic membrane (Gray 1975) and also seem to reach the post-synaptic density (Westrum and Gray 1976). This latter finding argues in favour of a functional continuity between tubulin in the post-synaptic density and the post-junctional lattice. Whilst Kelly and Cotman (1976) agree that tubulin is present at the synapse, they believe that the dominant protein at this location is actin because of the presence of 5 nm diameter fibres and electrophoretic evidence.

What emerges from all this is that finding alterations in colchicine binding can say very little about any <u>functional</u> role for tubulin in learning, and that what now needs to be tackled is whether and by what means the state of polymerization of tubulin and its compartmentation are changed by training. Measuring the degree of polymerization might be achieved fairly simply by estimating colchicine binding in the presence and absence of glycerol in the homogenate, as glycerol stabilizes microtubules. Because of the evidence of developmental modification of the competence of tubulin to polymerize, for whatever reason, in rat (Francon et al 1977), mouse (Koehn and Olsen 1978) and chick (Bamburg, Shooter and Wilson 1973) any training paradigms involving neonates are probably not the best to employ for such studies.

Even if the involvement of tubulin in imprinting is somewhat problematic, glycoproteins have been implicated since after one hour of exposure to an imprinting stimulus there is a 20% increase in ^{3}H-fucose incorporation in the anterior forebrain roof compared with dark birds (Dutton, Haywood and Hambley, unpublished data). This did not seem to be related to fucose availability. A number of interpretations are

possible. Fucosylation might occur to protein molecules present before exposure, or to those generated in response to the training experience, or to both. In any case the elevated glycosylation could have everything or nothing to do with learning, since the experimental design did not permit a dissociation between effects due to training or any of its contingencies such as light exposure. It is interesting however that ^{3}H-fucose incorporation is also elevated in the anterior forebrain roof of chicks trained on the one trial passive avoidance task (Mileusnic et al 1979).

In conclusion, the present experiments have shown that elevated incorporation of precursor occurs into a protein fraction of the anterior dorsal forebrain following a training experience in which the birds learn a preference for a familiar object, and that the degree of incorporation is related to the strength of the preference shown, which is taken as evidence that it is a learning related phenomenon. The significance of the anatomical localization of these changes will be assessed later.

TABLE 2 A

Preference scores (cm) for birds 27 cm from the imprinting stimulus

mean ± SEM (n)

	T1	T12
RED TRAINED	+2.9 ± 7.0 (23)	- 7.9 ± 6.1 (23)
YELLOW TRAINED	+3.9 ± 6.4 (22)	+10.7 ± 7.7 (22)

TABLE 2 B

Number of birds showing preference for the familiar (percentage of total)

	T1	T12
RED TRAINED	43	26
YELLOW TRAINED	55	45

TABLE 3

Incorporation of ^{14}C-lysine into acid insoluble (AI) and acid soluble (AS) fractions of anterior dorsal forebrain. Specific radioactivities (cpm mg^{-1} protein) mean $\pm$ SEM (n)

FRACTION	STIMULUS EXPOSED	DARK
AI	631 $\pm$ 63 (14)	593 $\pm$ 52 (14)
AS	745 $\pm$ 47 (14)	857 $\pm$ 57 (14)

TABLE 4

Incorporation of ^{14}C-lysine into AI and AS fractions of anterior forebrain roof after varying periods of exposure to an imprinting stimulus. Specific radioactivities (cpm mg^{-1} protein) mean ± SEM (n)

FRACTION	TIME (min)	STIMULUS EXPOSED	DARK
AI	30	573 ± 63 (8)	517 ± 56 (7)
	45	477 ± 51 (10)	440 ± 26 (7)
	90	427 ± 39 (6)	423 ± 77 (6)
AS	30	916 ± 51 (8)	897 ± 104 (7)
	45	933 ± 107 (9)	829 ± 41 (8)
	90	845 ± 48 (6)	947 ± 111 (5)

TABLE 5

Acid insoluble radioactivity in chick brain regions after varying periods of exposure to an imprinting stimulus. Specific radioactivities (cpm mg^{-1} protein) mean ± SEM (n)

TIME (min)	STIMULUS EXPOSED				DARK			
	anterior	posterior	base	midbrain	anterior	posterior	base	midbrain
30	710 ± 103 (8)	688 ± 109 (8)	694 ± 124 (8)	536 ± 88 (8)	677 ± 50 (8)	615 ± 50 (7)	639 ± 36 (8)	536 ± 44 (7)
60	613 ± 94 (12)	514 ± 82 (12)	549 ± 81 (12)	456 ± 66 (12)	512 ± 35 (12)	463 ± 34 (12)	454 ± 33 (12)	374 ± 26 (12)
90	497 ± 44 (11)	449 ± 46 (11)	436 ± 46 (11)	348 ± 52 (10)	504 ± 56 (11)	440 ± 52 (11)	455 ± 50 (11)	381 ± 42 (11)

TABLE 6

Acid soluble radioactivity in chick brain regions after varying periods of exposure to an imprinting stimulus. Specific radioactivities (cpm mg^{-1} protein) mean ± SEM (n)

TIME (min)	STIMULUS EXPOSED				DARK			
	anterior	posterior	base	midbrain	anterior	posterior	base	midbrain
30	797 ± 48 (9)	812 ± 46 (9)	964 ± 62 (9)	1130 ± 76 (9)	764 ± 46 (9)	731 ± 48 (8)	893 ± 66 (9)	1091 ± 129 (8)
60	765 ± 47 (12)	746 ± 48 (12)	916 ± 65 (12)	992 ± 72 (12)	742 ± 27 (12)	733 ± 38 (12)	883 ± 40 (12)	987 ± 44 (12)
90	796 ± 51 (10)	814 ± 64 (10)	994 ± 96 (10)	1009 ± 50 (10)	795 ± 57 (10)	714 ± 55 (10)	849 ± 57 (10)	958 ± 53 (10)

TABLE 7 A

Preference scores (cm) for birds 50 cm from the imprinting stimulus mean ± SEM (n)

	T1	T12
RED TRAINED	+5. 5 ± 3.9 (22)	+12.6 ± 8.1 (22)
YELLOW TRAINED	+8.13 ± 3.9 (22)	+ 9.3 ± 7.7 (22)

TABLE 7 B

Number of birds showing preference for the familiar (percentage of total)

	T1	T12
RED TRAINED	60	60
YELLOW TRAINED	50	60

TABLE 8

Acid insoluble fractions in chick brain regions. Stimulus-chick distance 50 cm. Specific radioactivities (cpm mg $^{-1}$ protein) means ± SEM (n)

TIME (min)	GROUP	anterior roof	posterior roof	forebrain base	midbrain
30	EP	619 ± 33 (8)	566 ± 69 (8)	551 ± 70 (8)	464 ± 61 (8)
	ED	633 ± 65 (7)	605 ± 61 (7)	552 ± 67 (7)	501 ± 74 (7)
	DP	640 ± 110 (7)	590 ± 97 (7)	567 ± 96 (7)	476 ± 86 (7)
	DD	636 ± 70 (8)	583 ± 90 (8)	505 ± 60 (8)	464 ± 63 (8)
60	EP	645 ± 99 (8)	551 ± 71 (8)	492 ± 66 (8)	417 ± 59 (8)
	ED	660 ± 71 (8)	638 ± 76 (8)	581 ± 72 (8)	491 ± 52 (8)
	DP	685 ± 80 (8)	559 ± 67 (8)	516 ± 86 (8)	429 ± 64 (8)
	DD	513 ± 47 (8)	485 ± 48 (8)	440 ± 43 (8)	390 ± 35 (8)

TABLE 9

Acid soluble fractions in chick brain regions. Stimulus-chick distance 50 cm. Specific radioactivities (cpm mg^{-1} protein) means ± SEM (n)

TIME (min)	GROUP	anterior roof	posterior roof	forebrain base	midbrain
30	EP	769 ± 43 (8)	763 ± 42 (8)	919 ± 29 (8)	984 ± 41 (8)
	ED	710 ± 65 (7)	750 ± 50 (7)	940 ± 46 (7)	984 ± 56 (7)
	DP	771 ± 34 (7)	842 ± 59 (7)	961 ± 44 (7)	918 ± 141 (7)
	DD	842 ± 56 (8)	797 ± 62 (8)	882 ± 72 (8)	994 ± 63 (8)
60	EP	744 ± 84 (8)	755 ± 60 (8)	870 ± 66 (8)	894 ± 66 (8)
	ED	715 ± 62 (8)	751 ± 66 (8)	869 ± 65 (8)	894 ± 81 (8)
	DP	758 ± 58 (8)	743 ± 49 (8)	886 ± 59 (8)	884 ± 67 (8)
	DD	651 ± 47 (8)	663 ± 36 (8)	788 ± 47 (8)	847 ± 49 (8)

TABLE 10

Preference scores (cm) for birds trained for one hour on an imprinting stimulus

		T0	T1	T24
RED TRAINED	(15)	+46.1 ± 8.2	+31.1 ± 7.7	+25.0 ± 6.1
YELLOW TRAINED	(16)	+25.8 ± 7.1	-11.4 ± 6.3	-20.5 ± 8.4

TABLE 11

Preference scores (cm) for birds trained for two hours on an imprinting stimulus

		T0	T1	T24
RED TRAINED	(15)	+13.2 ± 8.1	+16.8 ± 7.5	+22.1 ± 5.4
YELLOW TRAINED	(16)	-10.7 ± 7.3	-15.9 ± 6.9	+ 1 ± 8.2

TABLE 12

Preference scores (cm) of chicks trained for two hours either in a pen or a wheel

			T1	T24
RED TRAINED	PEN	(15)	+22.6 ± 5.91	+43.3 ± 10.0
	WHEEL	(15)	+49.3 ± 11.3	+42.5 ± 12.7
YELLOW TRAINED	PEN	(14)	-30.8 ± 8.4	+23.2 ± 6.3
	WHEEL	(15)	-26.2 ± 9.7	+ 9 ± 6.8

TABLE 13

Preference scores (cm) for birds used in ^{14}C-leucine incorporation study. Means ± SEM (n) birds.

GROUP	T1	T2
RED TRAINED	-1.4 ± 2.5 (21)	+6.25 ± 6.8 (20)
YELLOW TRAINED	+3.6 ± 2.4 (16)	+23.5 ± 8.0 (15)

TABLE 14

Two day ^{14}C-leucine incorporation experiment. Acid insoluble fractions. Standardised specific radioactivities (cpm mg^{-1} protein) mean ± SEM (n)

	DAY 1			DAY 2		
	DARK	RED TRAINED	YELLOW TRAINED	DARK	RED TRAINED	YELLOW TRAINED
ANTERIOR	1269 ± 112 (15)	1439 ± 141 (18)	1385 ± 188 (14)	1143 ± 95 (15)	1281 ± 66 (19)	1431 ± 123 (15)
POSTERIOR	1161 ± 91 (15)	1316 ± 124 (18)	1406 ± 168 (13)	1133 ± 89 (14)	1210 ± 75 (19)	1258 ± 80 (15)
BASE	1426 ± 153 (16)	1401 ± 131 (17)	1268 ± 171 (16)	1259 ± 125 (15)	1299 ± 81 (19)	1366 ± 122 (15)
MIDBRAIN	1429 ± 157 (14)	1603 ± 147 (17)	1389 ± 177 (16)	1206 ± 113 (14)	1397 ± 98 (19)	1577 ± 108 (14)

TABLE 15

Two day ^{14}C-leucine incorporation experiment. Acid soluble fractions. Standardised specific radioactivities (cpm mg^{-1} protein) mean ± SEM (n)

		ANTERIOR	POSTERIOR	BASE	MIDBRAIN
DARK REARED	DAY 1	556 ± 43 (14)	609 ± 60 (13)	753 ± 71 (13)	719 ± 68 (14)
	DAY 2	812 ± 66 (13)	758 ± 60 (14)	918 ± 71 (15)	935 ± 59 (13)
	t	3.28			2.38
	p	0.01	n.s	n.s	0.05
RED TRAINED	DAY 1	562 ± 51 (17)	506 ± 40 (17)	728 ± 58 (16)	733 ± 57 (16)
	DAY 2	900 ± 73 (19)	845 ± 80 (19)	1003 ± 69 (19)	1015 ± 68 (19)
	t	3.53	3.65	2.96	3.10
	p	0.01	0.001	0.01	0.01
YELLOW TRAINED	DAY 1	625 ± 85 (14)	631 ± 88 (13)	729 ± 64 (16)	795 ± 73 (16)
	DAY 2	807 ± 77 (13)	771 ± 45 (13)	939 ± 76 (11)	1177 ± 159 (11)
	t			2.09	2.43
	p	n.s	n.s	0.05	0.05

TABLE 16

Two day ^{14}C-leucine incorporation experiment. Disc retained acidic fraction. Standardised specific radioactivities (cpm mg^{-1} protein) mean ± SEM (n)

	DAY 1			DAY 2		
	DARK	RED TRAINED	YELLOW TRAINED	DARK	RED TRAINED	YELLOW TRAINED
ANTERIOR	1961 ± 165 (16)	1672 ± 127 (19)	1868 ± 186 (16)	1545 ± 105 (16)	1648 ± 81 (19)	2020 ± 187 (15)
POSTERIOR	1686 ± 160 (16)	1653 ± 166 (19)	1851 ± 192 (15)	1613 ± 181 (16)	1484 ± 74 (21)	1998 ± 197 (16)
BASE	1588 ± 144 (16)	1602 ± 130 (19)	1753 ± 193 (16)	1395 ± 119 (15)	1408 ± 61 (20)	1633 ± 133 (15)
MIDBRAIN	1551 ± 117 (16)	1690 ± 133 (19)	1785 ± 196 (16)	1483 ± 112 (16)	1549 ± 87 (21)	1781 ± 105 (15)

TABLE 17

Preference scores (cm) of birds used in the colchicine binding study. Mean ± SEM (n)

RED TRAINED	+28.09 ± 10.67 (11)
YELLOW TRAINED	+39.25 ± 10.63 (12)

TABLE 18

Two day colchicine binding experiment ^{3}H-colchicine binding. Specific radioactivities (dpm mg^{-1} protein) mean ± SEM (n)

		ANTERIOR	POSTERIOR	BASE	MIDBRAIN
DARK REARED	DAY 1	24086 ± 555 (10)	24583 ± 856 (10)	22563 ± 923 (9)	23599 ± 786 (8)
	DAY 2	22635 ± 1047 (9)	20125 ± 1330 (9)	21061 ± 1173 (7)	20354 ± 1098 (7)
	t		2.88		2.45
	p		0.02		0.05
RED TRAINED	DAY 1	23710 ± 834 (12)	23943 ± 834 (12)	24283 ± 941 (11)	25152 ± 842 (11)
	DAY 2	21804 ± 1306 (11)	23691 ± 896 (11)	20969 ± 990 (10)	23048 ± 2102 (9)
	t			2.43	
	p			0.05	
YELLOW TRAINED	DAY 1	22949 ± 1399 (10)	24802 ± 808 (10)	24411 ± 857 (9)	24048 ± 896 (9)
	DAY 2	22952 ± 1118 (12)	21813 ± 807 (12)	20958 ± 873 (12)	22757 ± 953 (12)
	t		2.60	2.83	
	p		0.02	0.05	

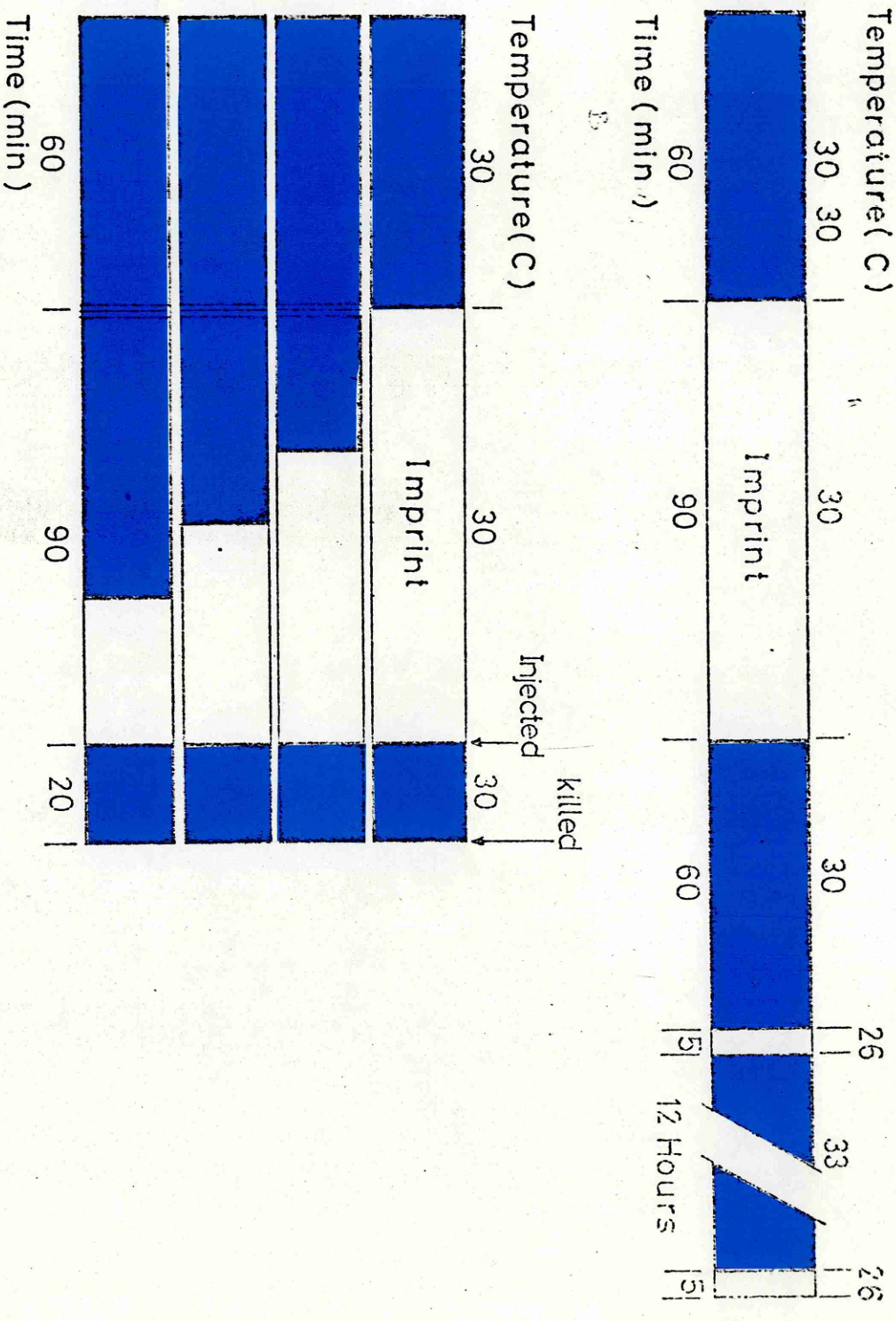

A
Temperature(C)
30 30
30
30
26
Imprint
60
90
60
5
33
12 Hours
26
5
Time (min)
B
Temperature(C)
30
30
Injected
killed
30
Imprint
B
60
90
20
Time (min)

Fig. 18

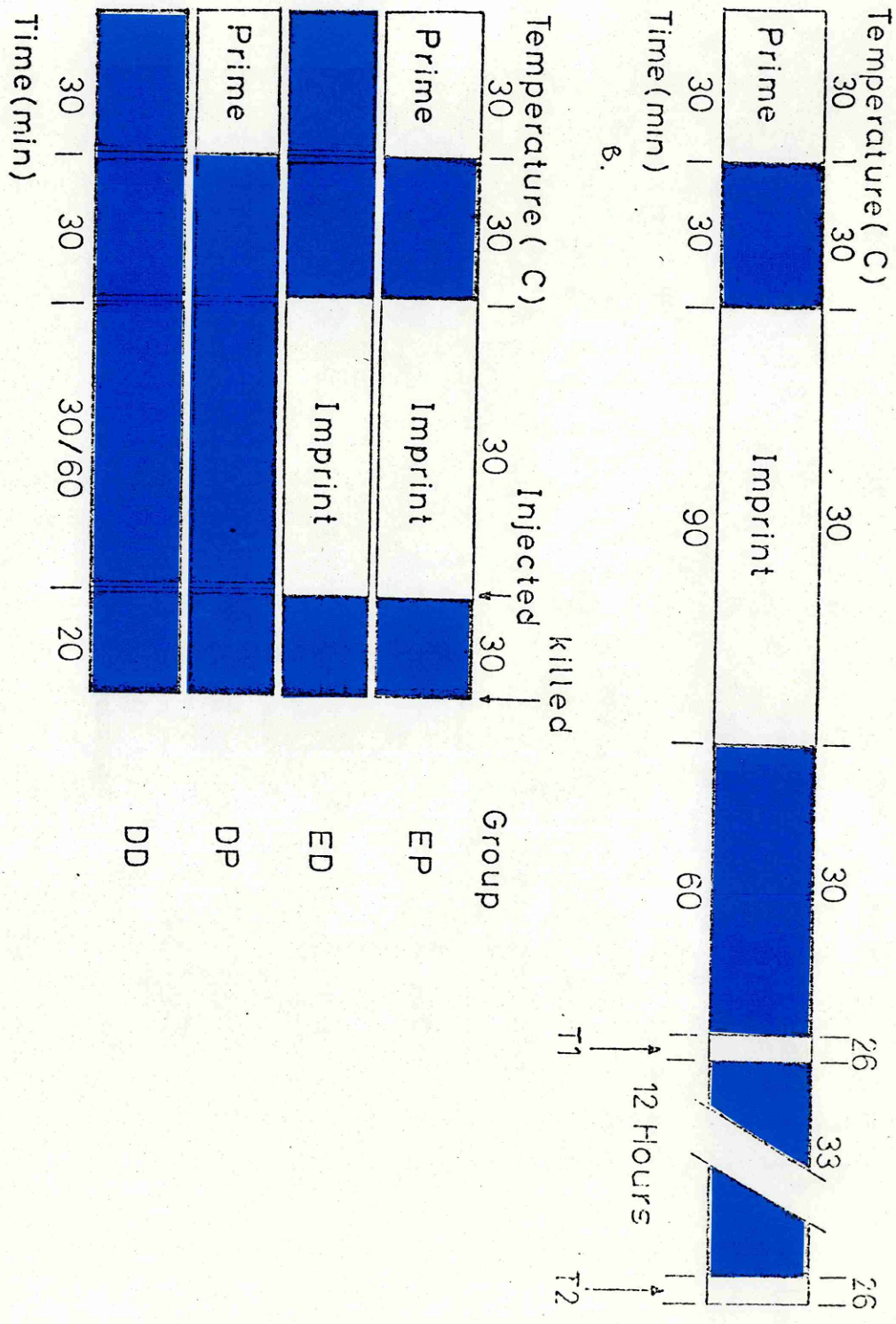

A
Temperature (C)
30
Prime
30
30
Imprint
30
26
33
26
Time (min)
30
30
90
60
12 Hours
T1
T2
B.
B
Temperature (C)
30
30
Injected
killed
Prime
Imprint
30
30
Group
EP
Prime
Imprint
ED
Prime
DP
DD
Time (min)
30
30
30/60
20

Fig. 19

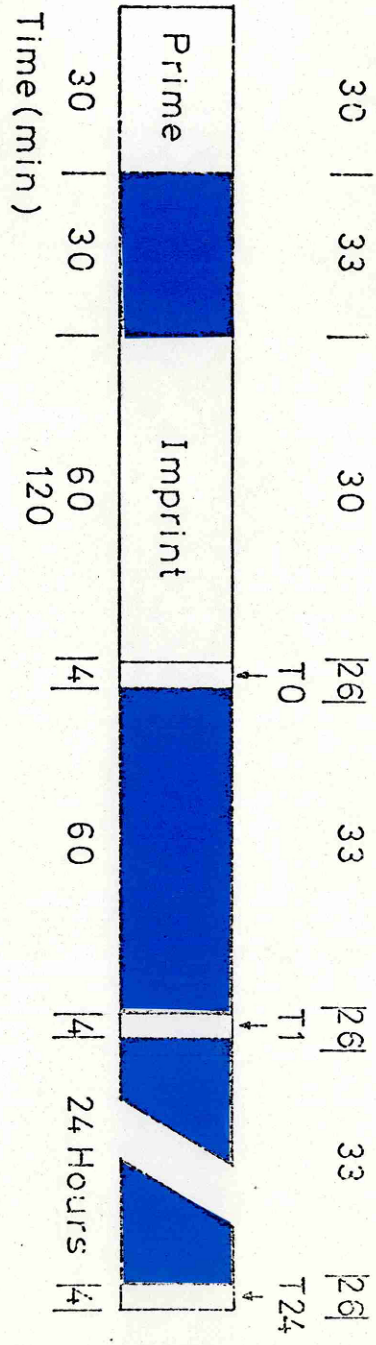

Temperature (C)
30
33
30
26
33
26
33
26
T0
T1
T24
Prime
Imprint
Time (min)
30
30
60
120
4
60
4
24 Hours
4

Fig. 20

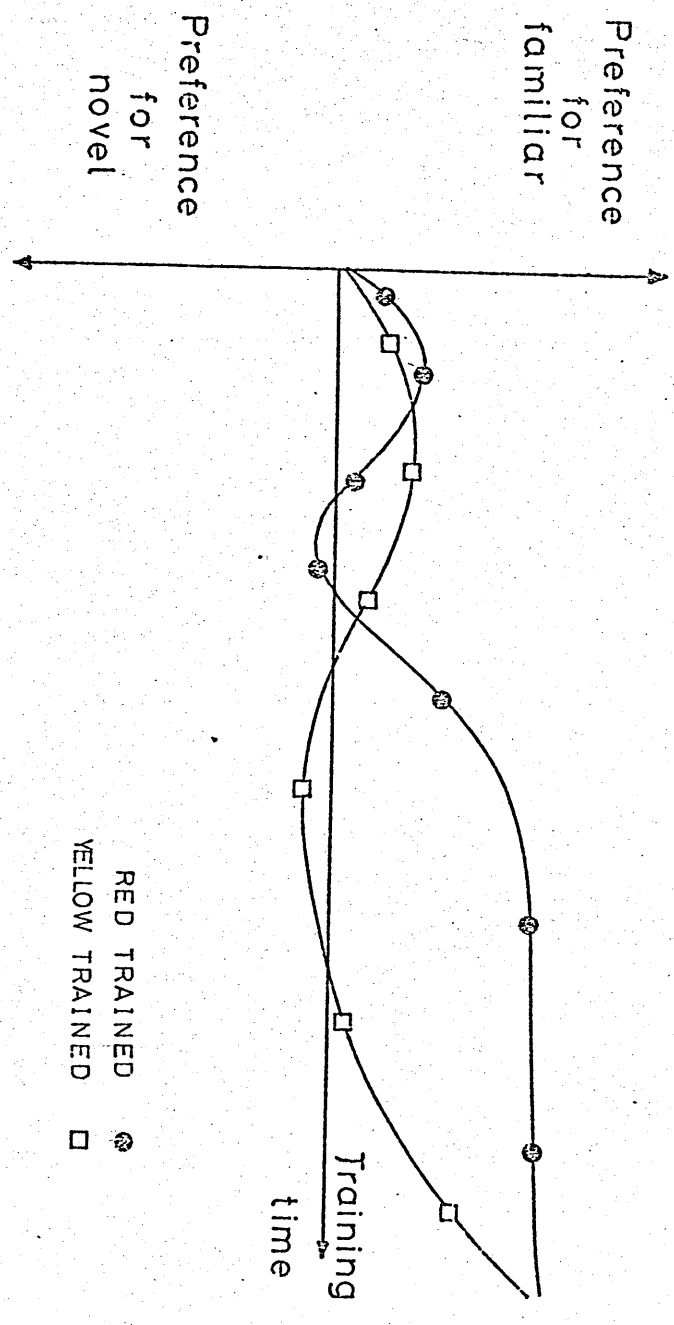

Preference for familiar
Preference for novel
Training time
RED TRAINED
YELLOW TRAINED

Fig. 21

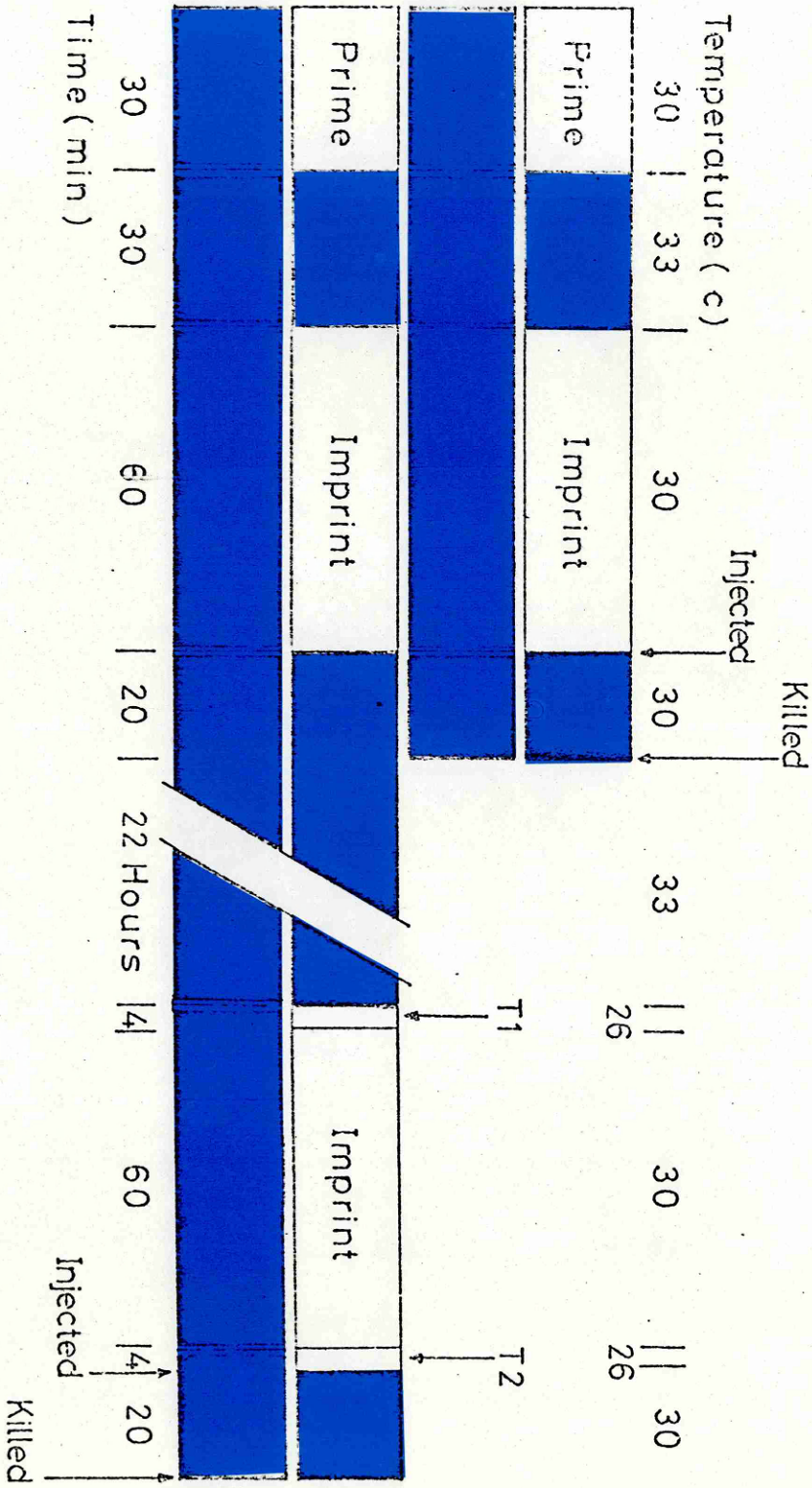

DAY 1
Temperature (c)
30
33
30
30
Injected
Killed
Prime
Imprint
33
26
30
26
30
T1
T2
Prime
Imprint
Imprint
Time (min)
30
30
60
20
22 Hours
4
60
4
20
Injected
Killed
DAY 2

Fig. 22

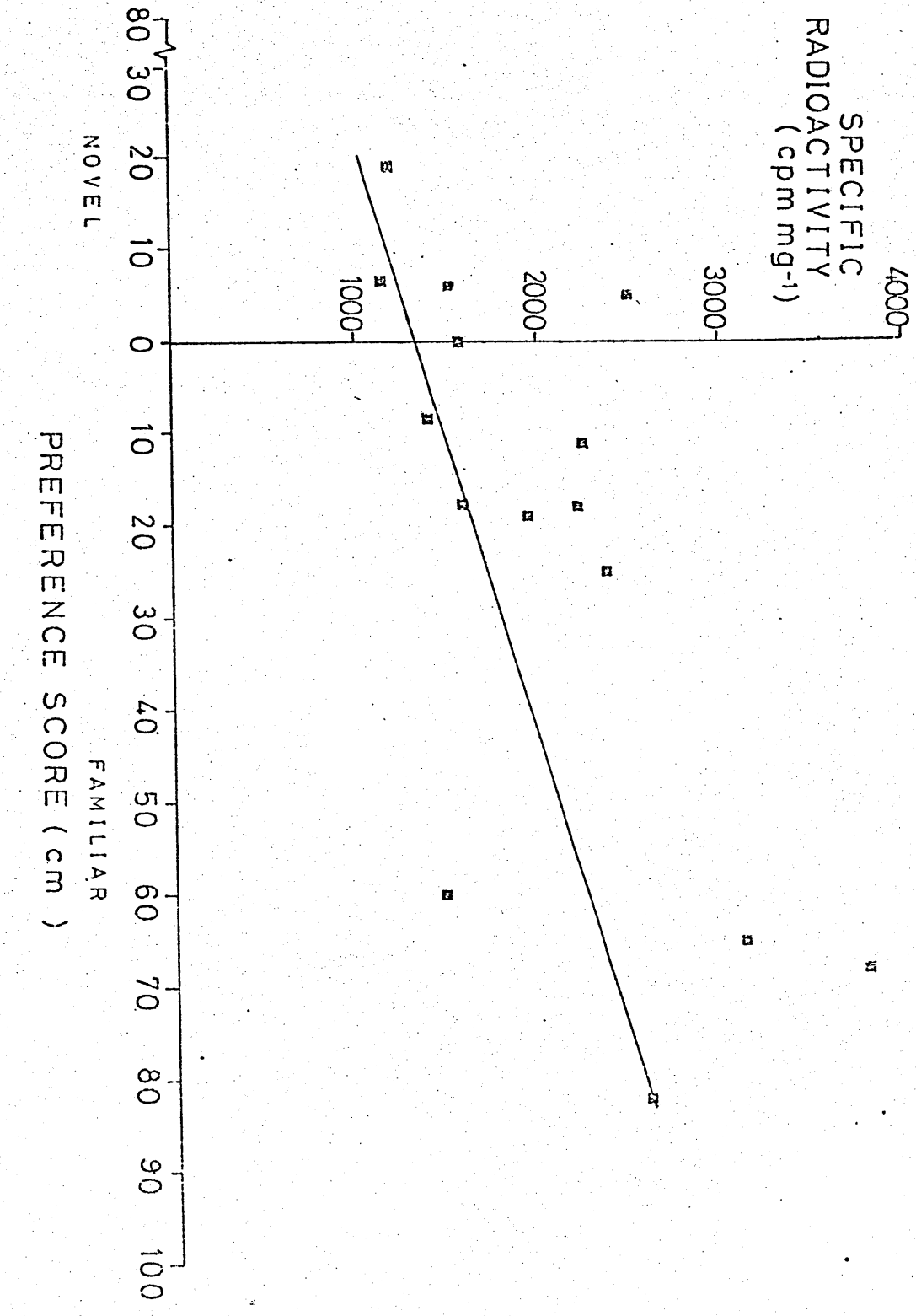

SPECIFIC
RADIOACTIVITY
(cpm mg-1)
1000
2000
3000
4000
80 30 20 10 0 10 20 30 40 50 60 70 80 90 100
NOVEL
FAMILIAR
PREFERENCE SCORE (cm)

Fig. 23

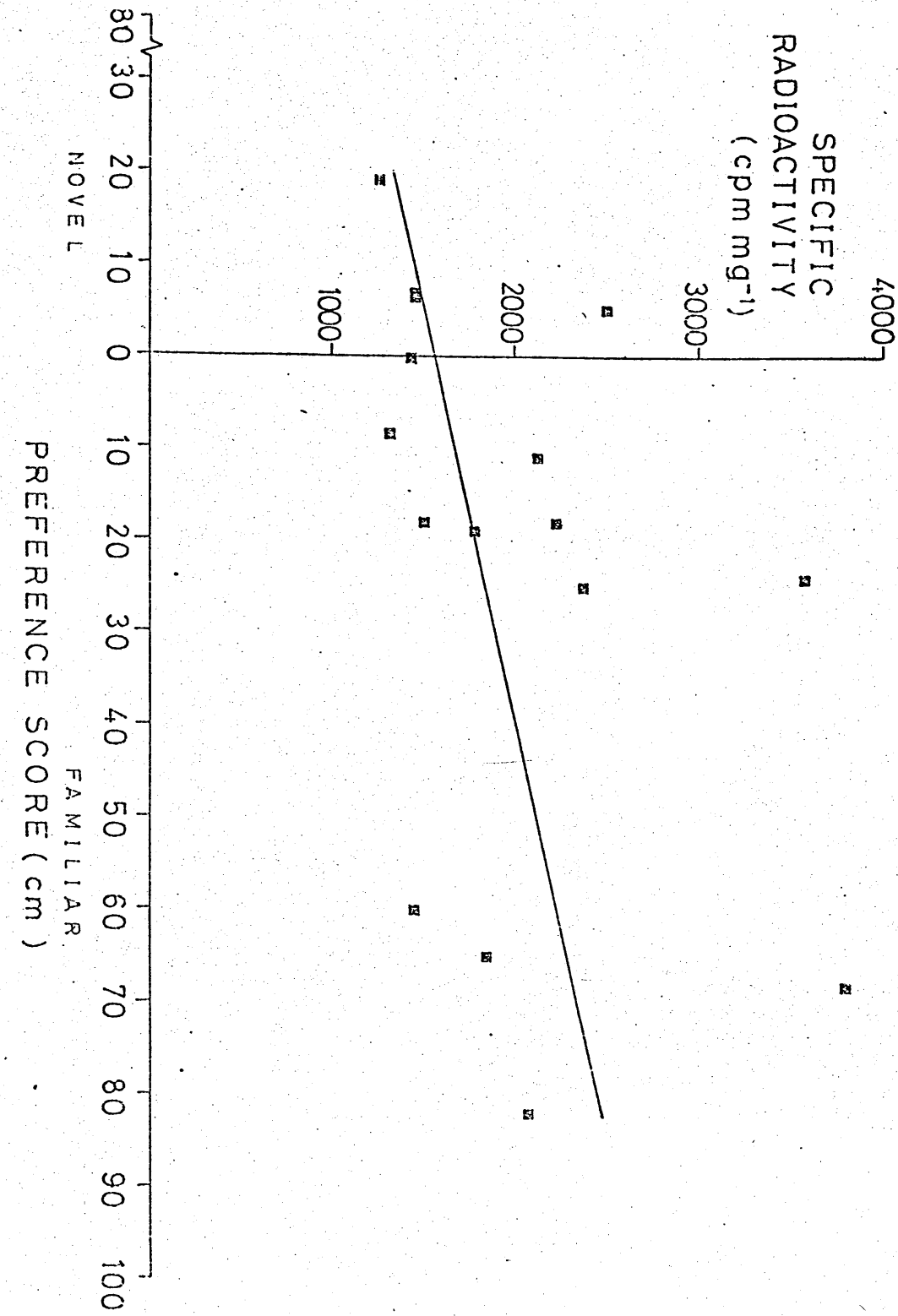

SPECIFIC
RADIOACTIVITY
(cpm mg⁻¹)
1000
2000
3000
4000
80 30 20 10 0 10 20 30 40 50 60 70 80 90 100
NOVEL
FAMILIAR
PREFERENCE SCORE (cm)

Fig. 24

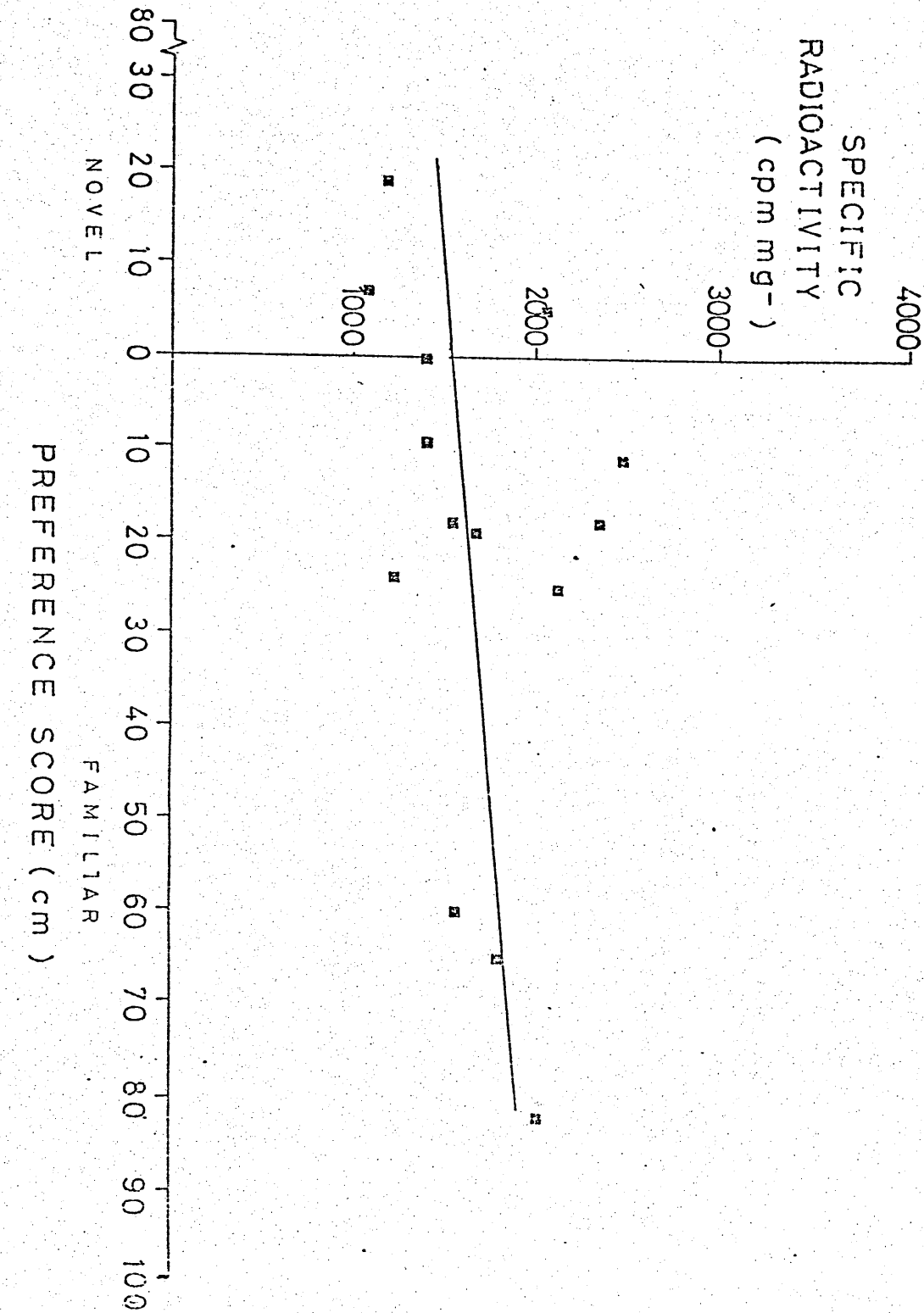

SPECIFIC
RADIOACTIVITY
(cpm mg⁻¹)
4000
3000
2000
1000
80 30 20 10 0 10 20 30 40 50 60 70 80 90 100
NOVEL
FAMILIAR
PREFERENCE SCORE (cm)

Fig. 25

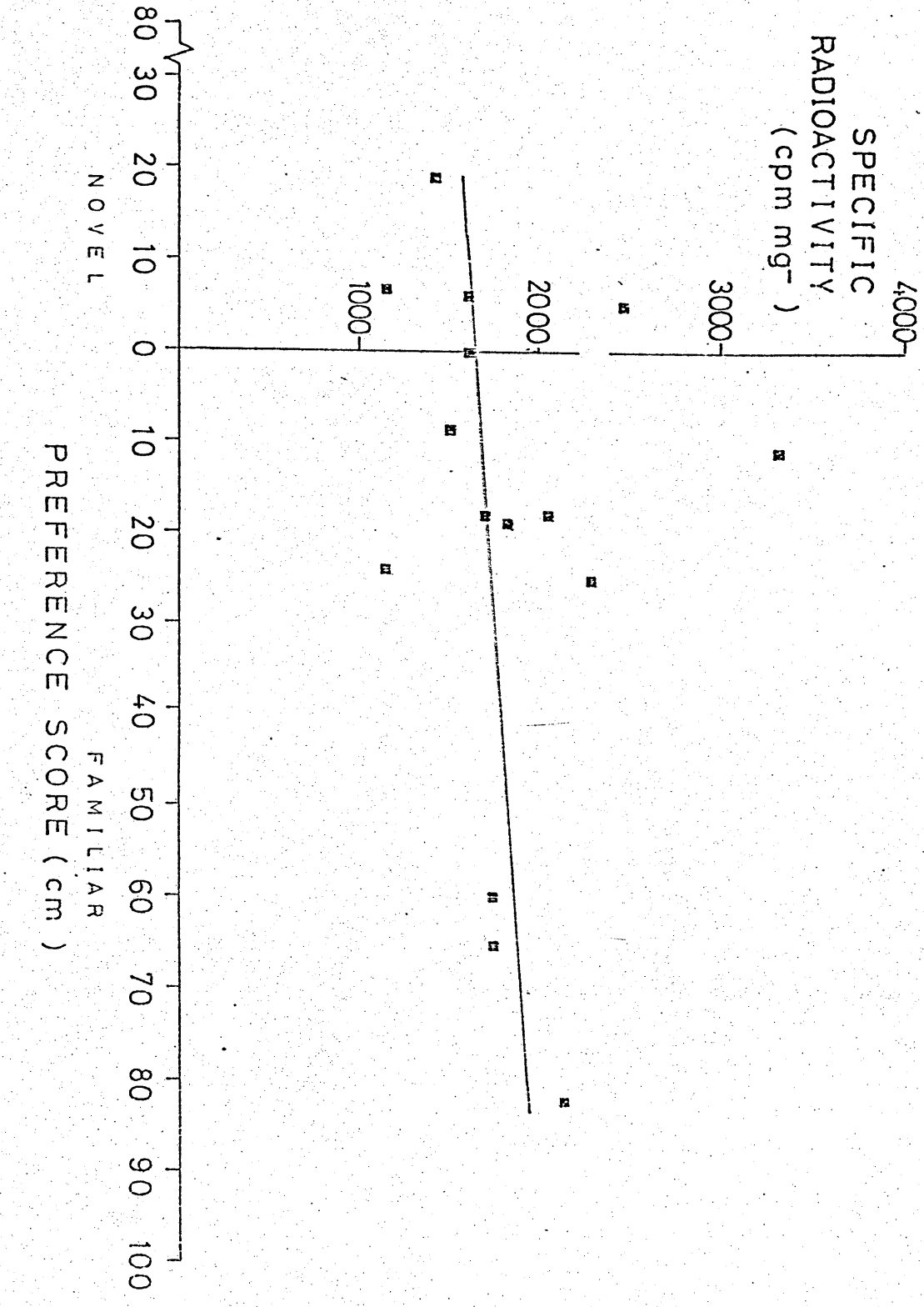

SPECIFIC
RADIOACTIVITY
(cpm mg⁻)
4000
3000
2000
1000
80 30 20 10 0 10 20 30 40 50 60 70 80 90 100
NOVEL
FAMILIAR
PREFERENCE SCORE (cm)

Fig. 26

LEGENDS

Table 2 A

Preference scores for birds 27 cm from stimulus. Results are given as the mean ± SEM of (n) birds. A positive score represents preference for the familiar, a negative score preference for novel.

Table 2 B

Number of birds showing preference for the familiar (percentage of total). T1 and T12 refer to results of a 5 minute test in the railway 1 and 12 hours after training, respectively.

Table 3

Acid insoluble (AI) and acid soluble (AS) specific radioactivities (cpm mg^{-1} protein) in the anterior dorsal forebrains of birds either exposed to an imprinting stimulus or kept in the dark for one hour, injected with ^{14}C-lysine and killed after 20 minutes. Results are given as mean ± SEM of (n) birds. The difference in the AI fraction between exposed and dark chicks is not significant.

Table 4

Incorporation of ^{14}C-lysine into AI and AS fractions of anterior dorsal forebrains of birds either exposed to an imprinting stimulus or kept in the dark for 30, 45 or 90 minutes. Results are given as the mean specific radioactivities (cpm mg^{-1} protein) ± SEM of (n) birds. There are no significant differences between exposed and dark birds at any time.

Table 5

Incorporation of ^{14}C-lysine into the acid insoluble fraction of chick brain regions after 30, 60 or 90 minutes exposure to an imprinting stimulus or being kept in the dark. Results are given as mean specific radioactivities (cpm mg^{-1} protein) ± SEM of (n) birds.

The longer the birds are left in the training chamber the less is the incorporation over the succeeding 20 minutes in all brain regions in the dark birds ($F = 68.84$, $p<0.05$) and in those exposed to the imprinting stimulus ($F = 90.01$, $p<0.05$). In the dark birds there is no regional variation of precursor incorporation, whereas in

the stimulus exposed birds there is a consistent drop in incorporation with anterior roof posterior roof base midbrain ($F = 24.28$, $p < 0.025$). None of the differences between the exposed and dark birds is significant for any brain region at any time point.

Significance was estimated by 2 tailed 2 way analysis of variance.

Table 6

Incorporation of ^{14}C-lysine into the acid soluble fraction of chick brain regions after 30, 60 or 90 minutes exposure to an imprinting stimulus or being kept in the dark. Results are given as mean specific radioactivities (cpm mg $^{-1}$protein) $\pm$ SEM of (n) birds.

None of the differences between the exposed and dark birds is significant for any brain region at any time point.

Table 7 A

Preference scores for birds 50 cm from an imprinting stimulus. Results are given as mean $\pm$ SEM of (n) birds. A positive score represents preference for the familiar, a negative score preference for novel.

Table 7 B

Number of birds showing preference for the familiar (percentage of total).

T1 and T12 refer to the results of a 5 minute test in the railway 1 and 12 hours after training, respectively.

Table 8

Incorporation of ^{14}C-lysine into chick brain regions after either 30 or 60 minutes in the training chambers with or without exposure to the imprinting stimulus and with or without prior 'priming'. At both time points there was significant regional variation in the incorporation, with a decrease in rate occurring ventro-caudally across the brain, (at 30 minutes, $F = 74.47$, $p < 0.05$; at 60 minutes, $F = 33.95$, $p < 0.05$). No significant differences occurred at either time between trained groups.

Significance estimated by 2 tailed, 2 way analysis of variance.

Table 9

Free radioactivity in chick brain regions from the experiment detailed in the previous legend.

Regardless of the treatment received or the time spent in the training chambers marked regional variation in the acid soluble fraction can be seen, the tendency being for higher levels of free lysine in the ventral telencephalon and midbrain than the dorsal forebrain (at 30 minutes, $F = 19.33$, $p<0.025$; at 60 minutes, $F = 120.59$, $p<0.05$). Again the different treatments appeared to have no significant effect.

Significance estimated by 2 tailed, 2 way analysis of variance.

Table 10

Preference scores (cm) for birds trained for one hour on an imprinting stimulus and given a 5 minute test on the railway immediately (T0) after one hour (T1) or 24 hours (T24). Results are given as means $\pm$ SEM of (n) birds. A positive score indicates preference for the familiar, a negative score preference for novel.

Table 11

Preference scores (cm) for birds trained for two hours on an imprinting stimulus. Other details are identical to the previous legend.

Table 12

Preference scores (cm) of chicks trained for two hours either in a pen or a railway. Note that in this experiment birds were not tested immediately after the conclusion of training. All other details are identical to the legend for Table 11.

Table 13

Preference scores (cm) for day 2 birds. T1 and T2 are separated by a one hour training session on either a red or a yellow imprinting stimulus as described in the text. Results are given as the means $\pm$ SEM of (n) birds. A positive score represents preference for the familiar, a negative score preference for novel.

The difference between red and yellow trained chicks at T2 is significant at $p=0.05$ level. (Kolmogorov-Smirnov 2 sample test).

Table 14

Incorporation of ^{14}C-leucine into acid insoluble fractions of dark and trained birds. Results are calculated as standardised specific radioactivities (cpm mg $^{-1}$protein) and expressed as means ± SEM of (n) birds.

Table 15

Ontogenetic changes in acid soluble fractions from dark and trained birds either 21 or 43 hours post-hatch. Results are calculated as standardised specific radioactivities (cpm mg $^{-1}$protein) and expressed as the means ± SEM of (n) birds.

Significance estimates are by unpaired, 2 tailed t test for 2 means.

Table 16

Changes in incorporation of ^{14}C-leucine into DEAE cellulose disc retained proteins of dark and trained birds. Results are calculated as standardised specific radioactivities (cpm mg $^{-1}$protein) and expressed as the means ± SEM of (n) birds. For the anterior roof of the dark birds D1/D2 = 1.27, t = 2.13, $p<0.05$. Significance estimated by unpaired, 2 tailed t test for 2 means.

For differences between groups on day 2:

	Ratio	F	p <
Anterior roof D v R v Y		3.72	0.05
Y/D	1.31	6.70	0.01
Y/R	1.23	4.45	0.05
Posterior roof D v R v Y		3.20	0.05
Y/D	1.24	3.05	n.s
Y/R	1.34	6.16	0.01

Significance estimates by one way analysis of variance.

Table 17

Preference scores (cm) for day 2 birds. Birds tested after a one hour training session on either a red or yellow imprinting stimulus. Results are means ± SEM of (n) birds. A positive score represents preference for the familiar, a negative score preference for the novel.

Table 18

Ontogenetic changes in ^{3}H-colchicine binding of homogenates from dark and trained birds either 21 or 43 hours post-hatch. Results are calculated as specific radioactivities (dpm mg $^{-1}$protein) and expressed as the means ± SEM (n) birds. Significance estimates are by unpaired, 2 tailed t test for 2 means.

For differences between groups on day 2:

	Ratio	F	p <
Posterior roof D v R v Y		3.05	0.05
R/D	1.13	6.06	0.01

Significance estimated by one way analysis of variance.

Fig. 18 A

Experimental protocol used to investigate the behaviour of birds exposed to an imprinting stimulus in the original design of training equipment.

Fig. 18 B

Corresponding experimental protocol to prepare birds for ^{14}C-lysine incorporation study. For details see text.

Fig. 19 A

Experimental protocol used to investigate the behaviour of birds exposed to an imprinting stimulus in the modified training equipment.

Fig. 19 B

Corresponding experimental protocol used to prepare birds for study of ^{14}C-lysine incorporation study. For details see text.

Fig. 20

Experimental protocol used to investigate efficacy of one versus two hours of training, and of pen versus wheel training.

Fig. 21

Diagram to indicate hypothetical time course of the development of familiar preference when training on either red or yellow stimuli. For discussion see text.

Fig. 22

Protocol used for the two day experiments. Note that for the colchicine binding studies no T1 was performed.

Fig. 23

Correlation between preference scores of individual yellow birds and incorporation of ^{14}C-leucine into DEAE cellulose disc retained proteins of the anterior forebrain roof on day 2. Spearman Rank $r_s = 0.60$, $z = 2.26$, $p < 0.05$. The linear regression

was calculated by the least squares method.

Fig. 24

Plot of preference scores of individual yellow birds versus incorporation of ^{14}C-leucine into DEAE cellulose disc retained proteins of the posterior forebrain roof. The correlation was not significant for this region. The linear regression was calculated by the least squares method.

Fig. 25

Plot of preference scores of individual yellow birds versus incorporation of ^{14}C-leucine into DEAE cellulose disc retained proteins of the forebrain base. The correlation was not significant for this region. The linear regression was calculated by the least squares method.

Fig. 26

Plot of preference scores of individual yellow birds versus incorporation of ^{14}C-leucine into DEAE cellulose disc retained proteins of the midbrain. The correlation was not significant for this region. The linear regression was calculated by the least squares method.

CHAPTER 6

CHOLINGERGIC SYSTEM

6.1 Acetylcholinesterase light control study

Because of the finding of a number of alterations in cholinergic enzymes in chicks following exposure to an imprinting stimulus (Haywood, Hambley and Rose 1975), a likely candidate for a protein modified by learning might be acetylcholinesterase. An alternative explanation for the results of Haywood et al would be that light exposure per se had brought about the changes. These authors compared only two groups of animals, dark and stimulus exposed, and did not test the trained birds to determine whether the exposure had modified their preferences.

There were two obvious ways to test this hypothesis. One would be to replicate the earlier experiments, but include a control group of chicks which were simply exposed to diffuse light, with no experience of the imprinting stimuli, and the second would be to search for a correlation between the degree to which the birds had apparently learnt the stimulus characteristics as shown by their preference scores in a two way discrimination test. Both of these approaches were attempted.

For the first experiment chicks were hatched, collected and when 18-24 hours old 'primed' in a brooder at 30 C with 30 minutes exposure to diffuse overhead light and then returned to a dark brooder maintained at 33 C. Hatchmate chicks of the same age range which were to serve as dark controls were similarly moved to a dark brooder at 30 C for 30 minutes, and then transferred to a second dark brooder kept at 33 C. This procedure ensured identical handling for light exposed and dark maintained birds. Chicks were consigned randomly to one of three groups:-

1. Exposed to either a red or yellow imprinting stimulus (T)
2. Exposed in the imprinting chambers to light from a single overhead 40 W tungsten bulb (L)
3. Placed in a chamber with the stimulus in motor off/light off mode; dark maintained (D)

Chicks remained in their respective conditions for one hour and were then transferred to a dark brooder kept at 30 C for one hour, after which they were decapitated.

Anterior and posterior forebrain roof regions were dissected out and these were

placed in coded vials on ice, and stored at -20 C until assayed.

Following homogenization in a glass-teflon homogenizer with 5 ml 155 mM NaCl the samples were assayed for AChE activity using the modification of the method of Ellman et al (1961) and estimated for total protein by the method of Lowry et al (1951), both as detailed in Chapter 4. The results were calculated as standardized specific activities (mkat kg^{-1} protein) and expressed as the means $\pm$ SEM of (n) birds.

These results are shown in Table 19 where it will be seen that in neither the anterior or posterior roof were there any marked differences between the three groups, suggesting that a one hour exposure to light has no effect on the activity of AChE in the chick forebrain one hour later. Possibly in this series of experiments differences due to training were not observed simply because the animals were failing to learn the characteristics of the stimulus. The correlation study was undertaken to test this.

6.2 Acetylcholinesterase correlation study

Chicks aged 24 $\pm$ 3 hours old were 'primed' with overhead diffuse light in a brooder kept at 30 C for 30 minutes. After a minimum of 30 minutes spent in a second brooder at 33 C birds were randomly assigned to be exposed for one hour to either a red or a yellow imprinting stimulus. Immediately following training birds were given a 5 minute test at 26 C to determine their degree of preference for the familiar stimulus, and then returned to a dark brooder at 30 C for a further 55 minutes. The behavioural protocol is summarized in Fig 27. At the end of this period the birds were decapitated, their brains removed, dissected into the conventional four regions, each being placed into a coded vial on ice. Samples were transferred to a deep freezer where they were stored at -20 C until assayed.

Samples were homogenized with a glass-teflon homogenizer in 5 ml of 155 mM NaCl and 100 μl aliquots were removed for AChE assay and protein estimation.

For each brain region a scatter diagram was plotted of individual preference scores against the activity of the enzyme expressed as mkat kg^{-1} protein (Figs 28-31).

A linear regression analysis was done on the red trained birds for each brain region.

A striking difference was observed between the behaviour of those birds trained on the yellow stimuli, and those trained on the red. The former showed a marked preference for novelty, the latter a preference for the familiar (Table 20). In no brain region was any significant correlation detectable between the preference for the familiar and the specific activity of AChE.

6.3.1 Two day experiments: acetylcholinesterase activity

Although the previous experiment had failed to demonstrate any change in the activity of AChE in any brain region as a result of light exposure or the training experience it was thought possible that the two day behavioural experiment described previously would provide a further opportunity to look for any changes in AChE activity. At this stage an automated method was explored because of the large numbers of samples involved and was finally adopted because it gave better reproducibility than the manual assay. Pilot studies had established conditions for the assay done manually, and the features of the automated procedure were not essentially different from those of the manual method.

Using one way analysis of variance for comparisons between all three groups on day 1 and day 2, and 2 tailed t tests for two means to compare groups between day 1 and day 2 revealed no differences in any brain region. The results are summarized in Table 21.

That no differences could be detected between any condition of birds on either day strongly supports the view that this enzyme is not implicated in learning the characteristics of an imprinting stimulus, nor in the contingent sensory stimulation. The results of the light control and correlation studies reported earlier had also militated against the involvement of AChE in any aspect of imprinting behaviour. Moreover, no differences were observed in AChE activity between passive avoidance trained and untrained chicks of the same strain (Rose, Gibbs, and Hambley 1979). None of this rules out the possibility that changes at the cholinergic synapse might be engendered by a modification of the isoenzyme pattern of AChE, without there being increased total amounts of the enzyme.

Although earlier work has shown both transient and longer lasting changes in AChE activity in birds trained on an orange light by comparison with dark maintained controls (Haywood, Hambley and Rose 1975), it is worth noting that the behavioural

experimental apparatus and design were quite dissimilar. Moreover the assay in the present series of experiments has been modified since finding that phosphate caused an inhibition of the neonatal chick enzyme in vitro. Another explanation for the discrepancy between the present and earlier study might be genetic drift in the laying hens, and there are numerous examples of behavioural and biochemical differences between strains of the same species documented. Unsatisfactory though these post hoc considerations may seem, the failure to replicate the early work serves as a warning that the processes that behavioural neurochemistry sets out to examine, often using fairly gross tools, seem fragile and elusive because it is difficult to appreciate how altering apparently unimportant variables can have a radical effect on brain function.

That AChE is not implicated in these plastic modulation need not be too surprising since it may not be rate limiting for acetylcholine metabolism at the synapse, and it then becomes hard to appreciate exactly what fluctuating enzyme levels are effecting, especially as no co-factor, or inactive form of AChE is known (Turbow and Burkhalter 1968).

6.3.2 Two day experiments: muscarinic receptor population

Quite apart from modulations in the activity of enzymes involed in neurotransmitter metabolism another way in which the cholinergic pathways might respond plastically could be by an alteration in the population of post-synaptic cholinergic receptors. Certainly this could be one interpretation of the extensive pharmacological studies by Deutsch et al (see 1.10.13) which concluded that learning might be facilitated by a modification of the sensitivity of the post-synaptic membrane to AChE.

An attempt was made to investigate whether any change in muscarinic receptor could be attributable to the imprinting experience.

QNB is a specific irreversible ligand for muscarinic cholinergic receptor (mAChr) and the degree of binding is presumably a measure of the amount of such receptor in the tissue.

A number of pilot studies were conducted to determine the optimal conditions for measuring binding, and a description of these together with the results are summarized in Figs 15,16,17. Briefly these studies established that ^{3}H-QNB binding obeyed

saturation kinetics, was linear with respect to protein concentration, and at the concentration of ligand used the 'non-specific' binding was about half that of the atropine displaceable binding.

The results of the ^{3}H-QNB binding in the day 1/day 2 experiments are summarized in Tables 22 and 23. The discrepancy in the number of birds in the groups for these experiments arises for two reasons. Firstly a moderate number of samples awaiting only mAChr assay were inadvertently thawed in my absence and were discarded as unusable, and some samples gave poor triplicates and were discarded on this basis alone, before collating biochemical and behavioural data. Insufficient material remained for re-assay of these samples.

The first striking feature of these results is that there is regional heterogeneity in the distribution of ^{3}H-QNB binding. Generally the forebrain base and midbrain regions contain higher levels of binding than do the dorsal forebrain regions in dark and trained birds on both day 1 and day 2. When comparisons were made between regions using a 2 tailed t test for two means the majority of the differences in binding appeared highly significant (Table 23).

No significant differences were found in the binding in dark maintained birds between day 1 and day 2.

With the trained groups of birds, only in the posterior forebrain roof in those trained on the yellow stimulus was an alteration found over the first two days post-hatch, namely a large increase in binding between day 1 and day 2. The corollary to this is that on the second day the yellow trained birds have an increased binding in this region with respect to both red trained and dark birds. In the midbrain the reverse seems to be the case. For this region a significant reduction in ligand binding was observed in the yellow trained birds by comparison with the other two groups.

The large change in the posterior roof of yellow trained birds on day 2 is not simply a response to a longer period of light exposure (2 as opposed to 1 hour) as evidenced by the lack of any comparable effect in the red trained chicks. It cannot be a developmental change occurring over the first two post-hatch days as no alterations were seen in dark birds over this period. Indeed the trend for the dark birds is in the

opposite direction to that which would be predicted by an ontogenetic interpretation.

It will be recalled that after a single hour of training neither red nor yellow exposed chicks showed any change in mean preference scores 22 hours later; that is, they gave no impression of having become imprinted. In this regard it is noteworthy that no significant changes were seen between any of the groups in any anatomical locus on day 1. However, on the second day the mean preference scores of the yellow trained birds were far higher than the red trained. The yellow trained chicks, then, were special with respect to the other groups in that they demonstrated successful performance in a well validated test of learning. I would postulate then that the massive increase in mAChr in the posterior forebrain roof is evidence that mechanisms dependent on cholinergic processes are being modulated by the imprinting experience. These arguments apply equally well to the depression in receptor binding identified in the midbrain.

The increase in ^{3}H-QNB binding in the posterior dorsal forebrain of birds which exhibit good imprinting behaviour is in accord with findings in chicks of the same strain trained on a quite dissimilar learning paradigm. Thirty minutes after the acquisition of a task in which chicks learn to passively avoid an aversive taste stimulus, ^{3}H-QNB binding was found to be elevated in the whole forebrain (which includes the posterior roof). Moreover this elevation could not be generated by the aversive taste itself in the absence of learning. Administration of amnestic agents into the forebrain such as ouabain and CXM abolished the ^{3}H-QNB binding in the forebrain of trained chicks (Rose, Gibbs, and Hambley 1980)

The time course of the receptor modification in the midbrain is very rapid, the marked reduction is effected within 20 minutes of the conclusion of a one hour training session. Maybe masking of, or conformational changes to some of the receptors is taking place which subsequently alters ligand binding. Muscarinic receptors in mice and rats do not form a homogenous population, but have at least two interconvertible forms which differ in their affinity for agonists such as carbamylcholine. They show regional heterogeneity, that is, in the brain stem and hypothalamus over half of the receptors are in the high affinity state, whereas in the telencephalon and thalamus the majority have a low affinity. However Scatchard analysis of QNB binding isotherms shows that the affinity of both forms of the receptor for this antagonist are the same (Aronstam 1979). Were the same situation to

pertain in the neonatal chick brain, and if training resulted in a shift from a conformer with a high affinity for atropine, to one with a lower affinity, (although retaining unchanged affinity for QNB) the specifically bound QNB would be reduced. The alternative to conformational changes which limit binding is a rapid degradation unaccompanied by synthesis of new receptor.

Functionally it is not easy to see what the midbrain changes in mAChr represent. Alterations in AChE have been described in the optic tectum, such as the increased accumulation of a heavy, 11S, form of the enzyme between the time of hatching and day 15 post-hatch, which has been regarded as representing a maturational change correlated with the sensitive period for imprinting (Marchand, Chapouthier and Massoulie 1977). Autoradiographic studies have shown a high density of α-bungarotoxin in chick optic tectum (Polz-Tejera, Schmidt, and Karten 1975). There is a massive transient increase in such sites in both the tectum and retina at around the time of hatching (Wang and Schmidt 1976) when the visual system becomes functionally active. The later loss of binding sites was thought to correspond to maturation of cholinergic synapses in the visual system (Blozovski and Blozovski 1968). While suggestive, none of this can be taken as proof for a cholinergic avian retinotectal pathway, and there is considerable evidence to the contrary.

Enucleation in the neonatal chick has very little effect on the activity of AChE activity, choline uptake (Margolis and Bondy 1969) or choline acetyltransferase activity (Marchisio 1969) in the optic tectum; innervation does not appear to be a prerequisite for development of the cholinergic system, neither is the appearance of AChE a marker for synaptogenesis. The presence of AChE in this region from a very early age may merely reflect the fact that cholinergic elements occur in the history of all neuroblasts and does not mean that all such cells are destined to be cholinergic. It has been proposed that in the neuroblast AChE may be involved in the regulation of protein synthesis or the generation of phospholipid components in the cell membrane, and as histogenesis proceeds such a role becomes redundant (Filogama and Marchisio 1971).

Such negative evidence may not be particularly convincing, but more positive work has implicated glutamate as an avian visual transmitter. The denervated optic lobe of the chick shows depressed levels of glutamate and uptake of this amino acid compared with the normal contralateral lobe (Bondy and Purdy 1977a). The lesion is

probably neuronal as enucleation has no effect on glial cell number (Bondy and Margolis 1971) and glutamate uptake is high even before glial differentiation has taken place (Bondy and Purdy 1977b). The specificity of the uptake is further evidenced by its sodium dependency and localisation in the synaptosomal fraction.

Microiontophoretic studies show that glutamate is excitatory to a large proportion of pigeon tectal neurones and glutamate antagonists either reduced or abolished excitation generated by contralateral optic nerve electrical stimulation (Wang, Felix and Frangi1978). Taken together this evidence satisfies several of the criteria regarded as important in establishing a neurotransmitter role.

If glutamate is a visual system transmitter in the chick then a role for the cholinergic system is problematic though the visual system may have more than one neurotransmitter and it should be remembered that the midbrain contains brainstem areas that are not visual in function.

An involvement of the posterior dorsal forebrain which has not previously been implicated in any biochemical sequelae to imprinting is not necessarily surprising. The locus for the changes in RNA metabolism as observed by autoradiography, the medial hyperstriatum ventrale (MHV) (Horn McCabe and Bateson 1979) extends considerably into the posterior roof. Metabolic alterations in neuronal perikarya located in the anterior dorsal forebrain could clearly bring about synaptic modifications some distance away, in the posterior roof; either in a more caudal part of the MHV, or other areas, for example the hippocampus, a learning role for which can hardly be doubted. In fact, by injecting ^{3}H-leucine it has been possible to study the efferent projection of the MHV, some of which at least go to the archistriatum (Bradley and Horn 1979).

It is worthwhile emphasizing that the MHV is situated just caudal to the midpoint between the anterior and posterior poles of the forebrain; which is approximately where the cut is made to divide the dorsal forebrain into anterior and posterior parts. In retrospect then it seems fortuitous that much of the early work detected anterior roof changes specifically, and given that 'contamination' effects have been found in the posterior roof with regard to ^{14}C-leucine incorporation into the acidic protein fraction, it is just conceivable that other posterior roof effects, such as colchicine and QNB binding relate to a common anatomical locus, and that the

apparent differences in location reflect what was originally conceived as a fairly arbitrary dissection technique.

The importance of the MHV in early learning in the chick is becoming increasingly evident.

6.4. The medial hyperstriatum ventral and learning

In a one trial passive avoidance task chicks with bilateral ablations of the hyperstriatal complex were found to have severe learning deficits, while unilateral lesions produced considerably less impairment by comparison with unoperated controls (Benowitz 1972). The surgery did not prevent pecking by naive birds suggesting that vision, arousal and motivation were unaffected. Moreover large bilateral ablations to other regions, namely olfactory bulbs, nucleus accumbens, frontal hyperstriatum, paleostriatum, neostriatum did not produce similar learning deficits. Interestingly in the same study chicks learned the task equally well whether trained through the eye that was ipsilateral or contralateral to the surgery, but extinction could only be established by training the contralateral eye. The implication here is that the original learning could be transferred from one hemisphere to the other, whereas extinction could not; therefore extinction is not simply a reversal of original learning but a different process. Benowitz speculates that while the amygdala plays a part in the mediation of avoidance conditioning, and has connections to the contralateral hemisphere via which interhemispheric transfer can take place, the hippocampus, with no such connections, is involved in extinction processes.

Bilateral destruction of the MHV prevents the acquisition of a preference for the familiar without apparent damage to visuomotor co-ordination (Bateson, Horn and McCabe 1978). When neonatal chicks were exposed to a red flashing light they showed electrophysiological evidence of a greater responsiveness to visual stimulation in cells of the MHV and the hyperstriatum accessorium, (HA) which is a visual projection area, when compared with birds reared in the dark (Brown and Horn 1979). Also, in the HA a similar comparison revealed a 19 per cent increase in the volume of dendritic spines in the visually experienced animals (Bradley and Horn 1979). By injecting horseradish peroxidase it has been possible to trace inputs into the MHV from both the ipsilateral and contralateral optic tecta, indicating that it receives visual information directly from the midbrain, and it also has fibres from the HA (Bradley and Horn 1978), all of which clearly makes the

MHV well connected to mediate visually dependent behaviours.

In summary, chicks which develop a clear preference for the familiar following exposure to an imprinting stimulus show an increase in the amount of muscarinic receptor in the posterior dorsal forebrain, and such a change is quite consistent with the current knowledge concerning the functional anatomy of this region and the present thinking about the role of cholinergic mechanisms in learning.

TABLE 19

Acetylcholinesterase standardized specific activities (mkat kg^{-1} protein) mean ± SEM (n)

	D	L	T
Anterior roof	2.370 ± 0.119 (15)	2.509 ± 0.125 (15)	2.442 ± 0.122 (14)
Posterior roof	2.167 ± 0.094 (15)	2.306 ± 0.122 (15)	2.345 ± 0.094 (15)

TABLE 20

Acetylcholinesterase correlation study. Preference scores (cm) mean ± SEM (n)

YELLOW TRAINED	RED TRAINED
-12.6 ± 2.8 (39)	+17.0 ± 3.3 (47)

TABLE 21

Acetylcholinesterase activity. Standardized specific activities (mkat kg^{-1} protein) mean ± SEM (n)

	DAY 1			DAY 2		
	DARK	RED TRAINED	YELLOW TRAINED	DARK	RED TRAINED	YELLOW TRAINED
ANTERIOR	5.113 ± 0.25 (11)	5.145 ± 0.21 (13)	5.115 ± 0.24 (13)	4.439 ± 0.24 (12)	4.954 ± 0.22 (14)	4.368 ± 0.31 (11)
POSTERIOR	4.735 ± 0.20 (11)	4.903 ± 0.24 (13)	4.699 ± 0.21 (12)	4.457 ± 0.21 (12)	4.818 ± 0.22 (15)	4.392 ± 0.17 (11)
BASE	5.315 ± 0.40 (11)	5.720 ± 0.23 (13)	5.730 ± 0.35 (13)	5.388 ± 0.21 (12)	5.971 ± 0.28 (15)	5.294 ± 0.71 (11)
MIDBRAIN	6.770 ± 0.35 (11)	6.400 ± 0.26 (13)	5.926 ± 0.38 (13)	6.020 ± 0.25 (12)	6.457 ± 0.29 (15)	6.465 ± 0.23 (11)

TABLE 22

^{3}H-QNB binding. Standardized specific activities (fmoles mg $^{-1}$protein) mean ± SEM (n)

	DAY 1			DAY 2		
	DARK	RED TRAINED	YELLOW TRAINED	DARK	RED TRAINED	YELLOW TRAINED
ANTERIOR	119 ± 19 (12)	104 ± 13 (15)	154 ± 23 (12)	125 ± 16 (15)	132 ± 22 (15)	151 ± 29 (14)
POSTERIOR	102 ± 15 (12)	102 ± 17 (16)	95 ± 26 (17)	124 ± 16 (12)	91 ± 16 (14)	203 ± 43 (13)
BASE	237 ± 36 (15)	201 ± 23 (18)	160 ± 22 (15)	227 ± 29 (15)	235 ± 27 (17)	213 ± 24 (17)
MIDBRAIN	267 ± 35 (14)	249 ± 39 (17)	264 ± 35 (16)	307 ± 19 (14)	308 ± 15 (15)	198 ± 37 (16)

TABLE 23

^{3}H-QNB binding. Distribution throughout brain. Two tailed t tests for two means.

		DAY 1			DAY 2		
COMPARISON		DARK	RED TRAINED	YELLOW TRAINED	DARK	RED TRAINED	YELLOW TRAINED
Anterior v Base	t=	2.95	3.73	n.s	3.09	2.94	n.s
	p <	0.01	0.001		0.01	0.01	
Anterior v Midbrain	t=	3.79	3.54	2.6	7.28	6.5	n.s
	p <	0.001	0.01	0.02	0.001	0.001	
Posterior v Base	t=	3.46	3.41	n.s	3.12	4.6	n.s
	p <	0.01	0.01		0.01	0.001	
Posterior v Midbrain	t=	3.30	3.42	3.85	7.32	9.86	n.s
	p <	0.01	0.01	0.001	0.001	0.001	

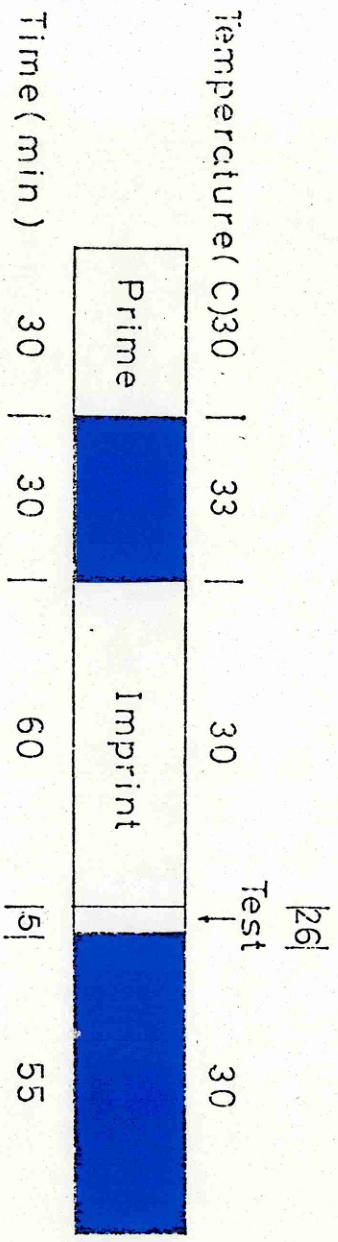

Temperature(C)30
33
30
26
Test
Prime
Imprint
Time(min)
30
30
60
5
30
55

Fig. 27

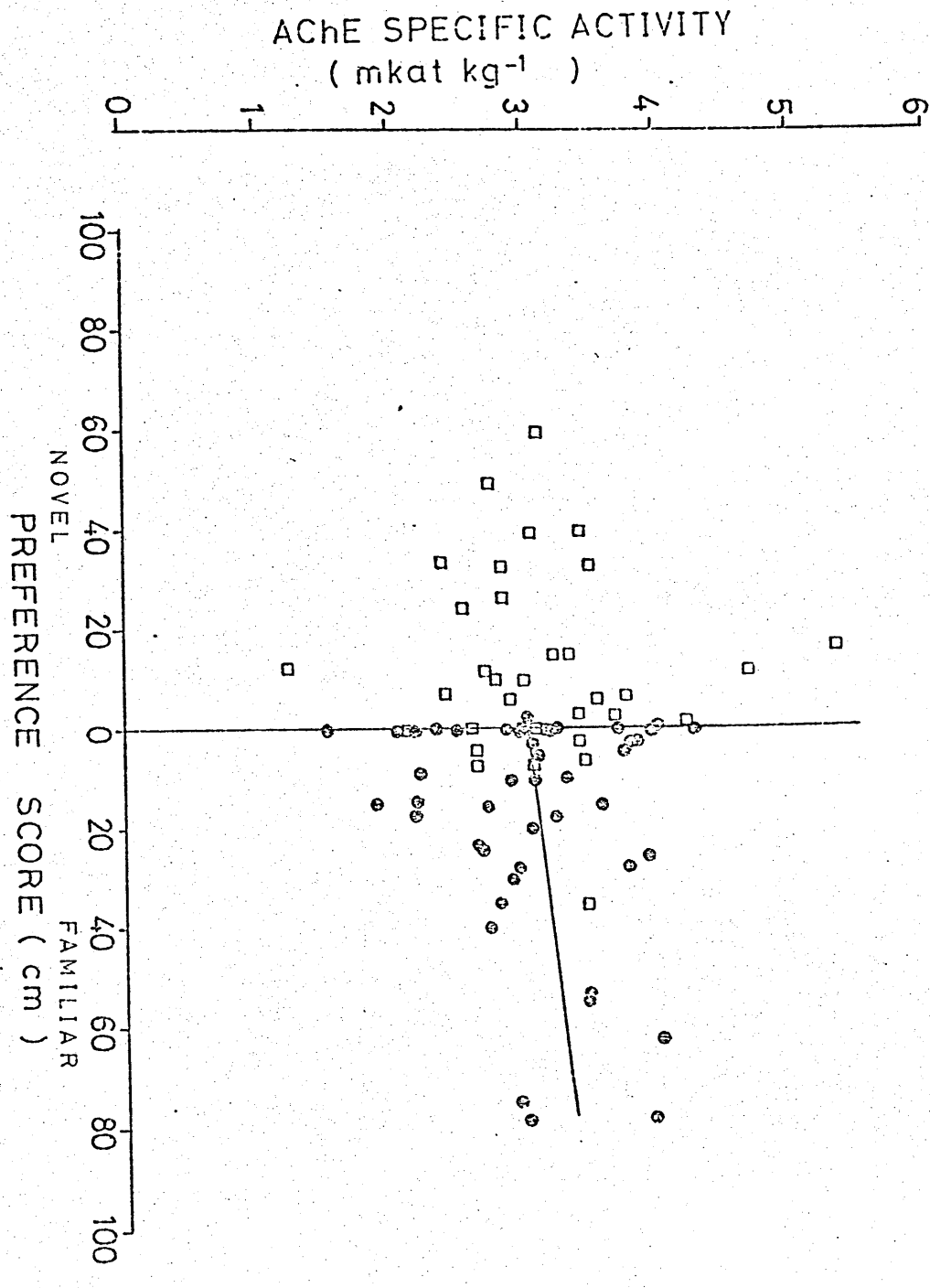

AChE SPECIFIC ACTIVITY
(mkat kg⁻¹)
0
1
2
3
4
5
6
PREFERENCE SCORE (cm)
NOVEL
FAMILIAR
100
80
60
40
20
0
20
40
60
80
100

Fig. 28

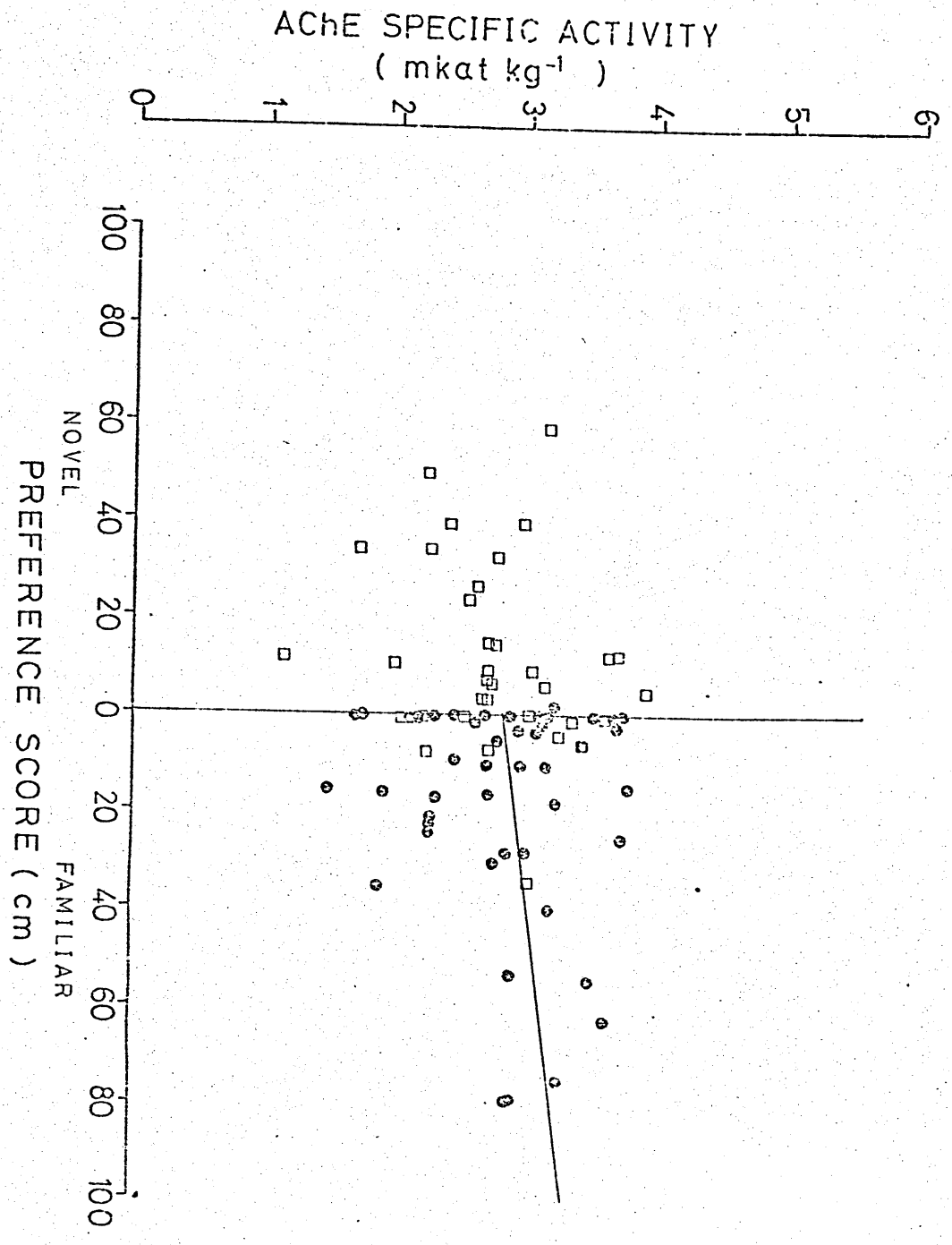

AChE SPECIFIC ACTIVITY
(mkat kg⁻¹)
0
1
2
3
4
5
6
100
80
60
40
20
0
20
40
60
80
100
NOVEL
PREFERENCE SCORE (cm)
FAMILIAR

Fig. 29

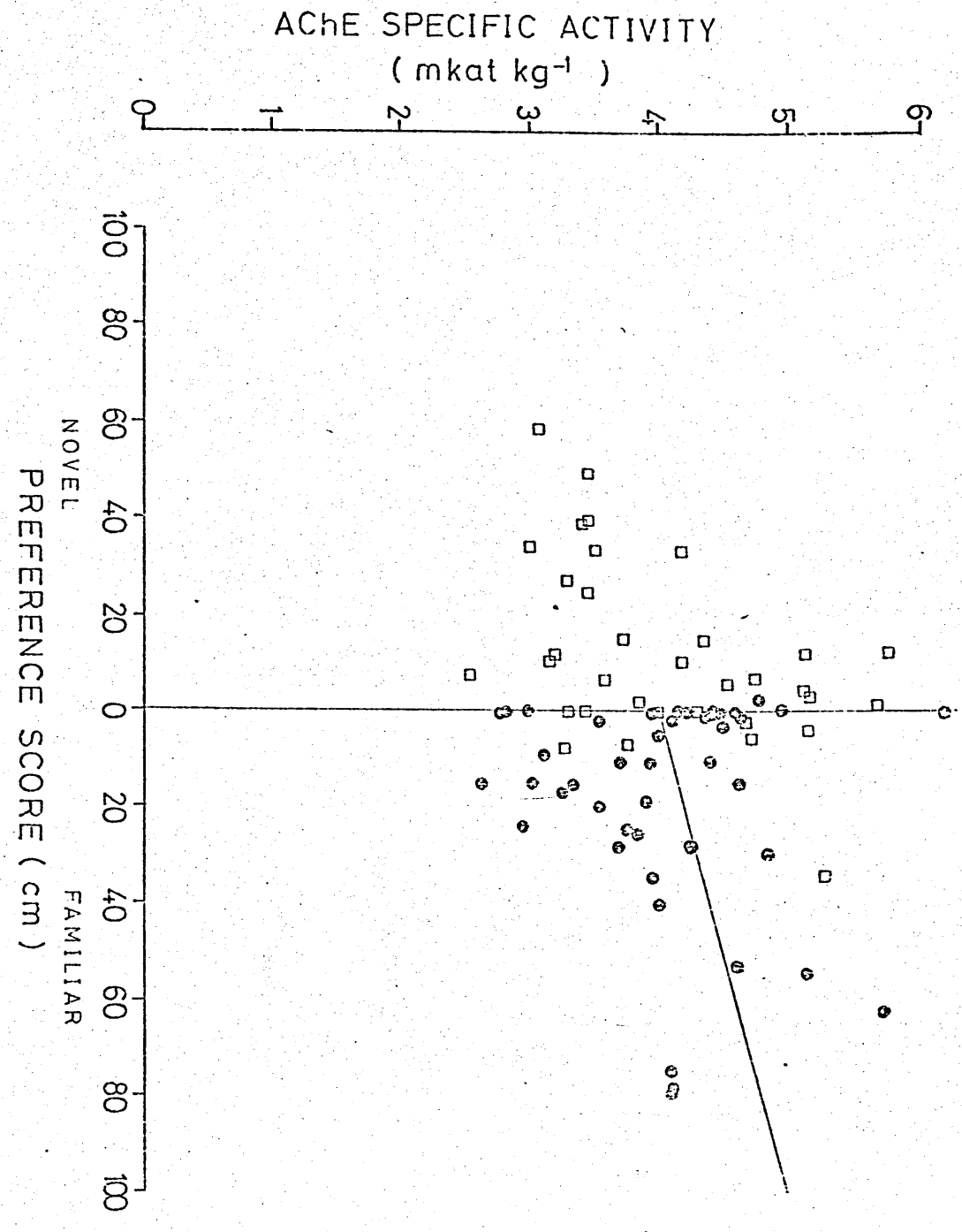

AChE SPECIFIC ACTIVITY
(mkat kg⁻¹)
0
1
2
3
4
5
6
PREFERENCE SCORE (cm)
NOVEL
FAMILIAR
100
80
60
40
20
0
20
40
60
80
100

Fig. 30

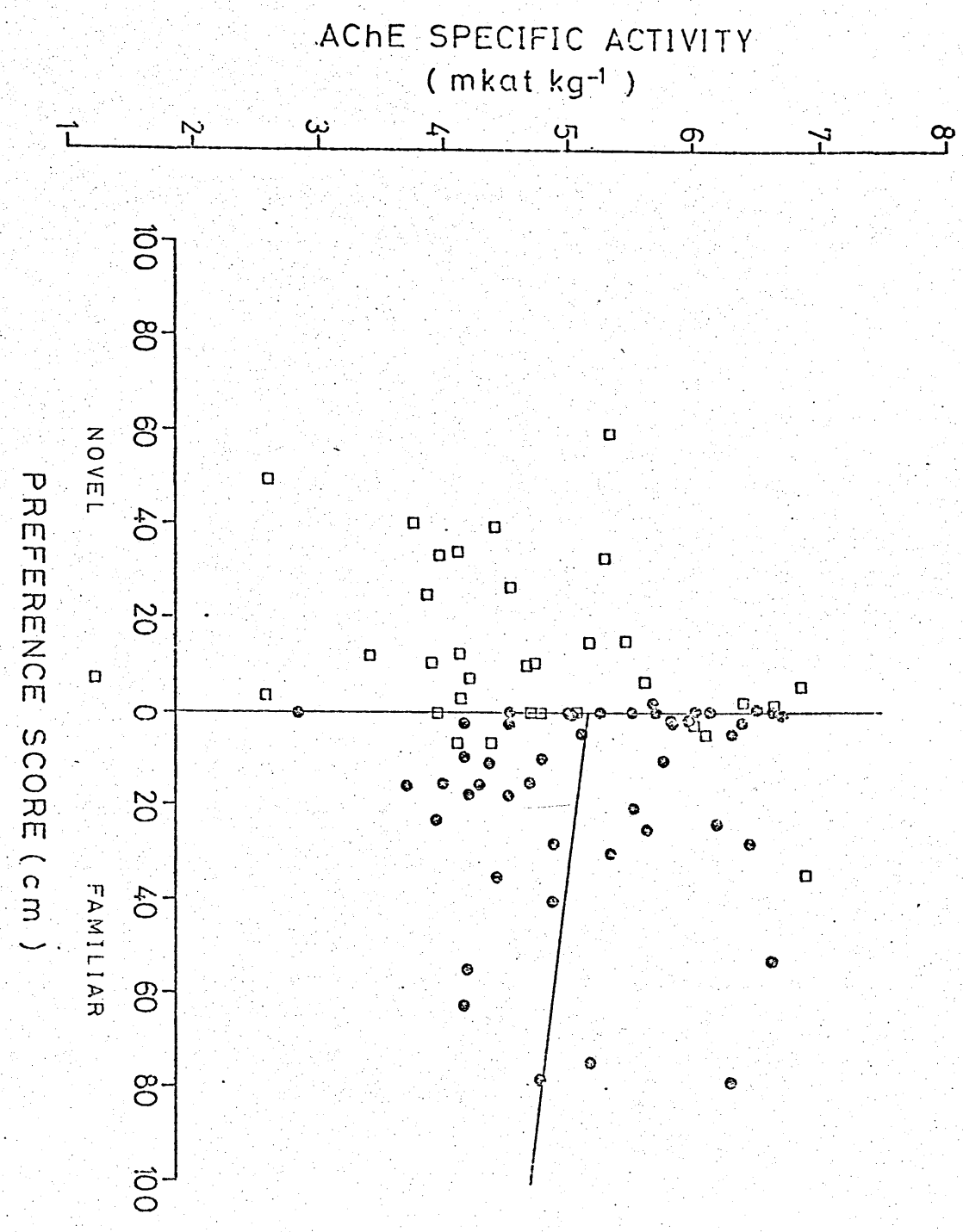

AChE SPECIFIC ACTIVITY
(mkat kg-1)
1
2
3
4
5
6
7
8
PREFERENCE SCORE (cm)
NOVEL
FAMILIAR
100
80
60
40
20
0
20
40
60
80
100

Fig. 31

Table 19

Acetylcholinesterase light control study. Chicks were either exposed to an imprinting stimulus (T) or a diffuse overhead light (L) or maintained in the dark (D) for one hour. One hour after their respective treatments birds were killed and dorsal forebrain regions assayed for AChE activity. Results are given as standardized specific activities (mkat kg^{-1} protein and expressed as means ± SEM of (n) birds.

Table 20

Acetylcholinesterase correlation study. Chicks trained by the behavioural protocol illustrated in Fig 27 were given a 5 minute test. The results of this test are given as preference score (cm) and expressed as means ± SEM of (n) birds. A positive score indicates a preference for the familiar, a negative score preference for the novel.

Table 21

Two day experiment: acetylcholinesterase study. AChE activity of homogenates from dark maintained and trained birds. Results are calculated as standardized specific activities (mkat kg^{-1} protein) and expressed as the means ± SEM of (n) birds.

Table 22

Changes in ^{3}H-QNB binding of homogenates from dark and trained birds. Results are calculated as standardized specific activities (fmoles QNB bound mg^{-1} protein) and expressed as the means ± SEM of (n) birds. Significance estimates are by one way analysis of variance.

For day 2 birds:	Ratio	F	p<
Posterior roof D v R v Y		4.05	0.05
Y/D	1.62	3.47	0.05
Y/R	2.07	7.82	0.01
Midbrain D v R v Y		5.02	0.05
Y/D	0.66	6.21	0.01
Y/R	0.64	7.89	0.01

Table 23

Statistical analysis of data given in Table 22, showing regional heterogeneity of ^{3}H-QNB binding. Significance estimated by 2 tailed t test for two means.

Fig. 27

Experimental protocol used in the acetylcholinesterase correlation study.

Fig. 28

Plot of preference scores (cm) of individual birds versus acetylcholinesterase activity (mkat kg^{-1}) in the anterior forebrain roof. The regression line for the red trained birds, which showed preference for the familiar, was calculated by the least squares method. There is no correlation between the variables.

Fig. 29

Plot of preference scores (cm) of individual birds versus acetylcholinesterase activity (mkat kg^{-1}) in the posterior forebrain roof. The regression line for the red trained birds was calculated by the least squares method.

Fig. 30

Plot of preference scores (cm) of individual birds versus acetylcholinesterase activity (mkat kg^{-1}) in the forebrain base. The regression line for the red trained birds was calculated by the least squares method.

Fig. 31

Plot of preference scores (cm) of individual birds versus acetylcholinesterase activity (mkat kg^{-1}) in the midbrain. The regression line for the red trained birds was calculated by the least squares method.

APPENDIX

The unit of enzyme activity adopted in this thesis is the katal, which is advocated by the Commission on Biochemical Nomenclature (1973), 1972 Recommendations on Enzyme Nomenclature, Elsevier, Amsterdam.

I katal (kat) will catalyse I mole sec^{-I} of substrate. All results are given in SI units, i.e. kat kg^{-I} proteins.

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